University of Trás-os-Montes e Alto Douro

Antimicrobial resistance, biofilm formation and cell invasion by bacteria isolated from wild animals: potential impact in animal and human health

PhD Thesis in Chemical and Biological Sciences

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“Nothing in life is to be feared, it is only to be understood”

Marie Curie

I dedicate this thesis to my parents, sister and to my goddaughter

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ABSTRACT

Antibiotics were originally developed for the treatment of bacterial infectious diseases. However, antibiotic resistance thwarts the treatment of infectious diseases globally, representing one of the most serious public health problems. The emergence of increasing numbers of antibiotic-resistant pathogens, some of which resistant to multiple classes of antibiotics, has implications not only for veterinary patients but also humans. Additionally, during the last years, wildlife has also been an important source of bacterial infectious diseases transmissible to humans. Zoonoses with a wild animal reservoir constitute a major public health concern worldwide, and the need for more attention in this area is consistently increasing. Bacteria can resist the action of antibiotics through several mechanisms, including antibiotic target modification, enzymatic inactivation or modification and prevention of access to antibiotic target. Another important factor that significantly contributes to increase antibiotic of resistance among pathogenic microorganisms is their ability to form biofilms. Biofilms are dynamic and complex systems, with characteristics of both primordial multicellular organisms and multifaceted ecosystems. Taking into consideration the public health concern on emerging antibiotic resistance, it becomes crucial to study the importance of wild animals as reservoirs of antibiotic resistant bacteria and their ability to form biofilms and interact with human cells.

The main goal of this study was to provide information about the presence and behavior of antibiotic resistant bacteria from selected wild animals of the North of Portugal. Therefore, bacteria isolated from wild animals were selected for identified, and characterized for their antibiotic susceptibility profile, in both planktonic and sessile states.

The ability of some strains to survive under human gastrointestinal conditions was evaluated. Moreover, their ability to attached/internalized the human colon adenocarcinoma cells line (Caco-2), and produced virulence factors, namely *N*-acyl homoserine lactones (AHLs), siderophores, proteases and gelatinases was also assessed.

The identification of 20 strains was accomplished by phenotypic analysis, and 16s rDNA and *gyr*B sequencing. Sixteen strains were identified as belonging to the genus *Aeromonas*. On the basis of *gyr*B sequence alignments it was observed that the strains were clustered in 4 phylogenetic groups with 9 *gyr*B different sequences: *Aeromonas salmonicida*, *A. eucrenophila*, *A. bestiarum* and *A. veronii*. The remaining strains belong to the genera *Acinetobacter*, *Pseudomonas*, *Klebsiella* and *Shewanella.* The study of the bacterial

antimicrobial susceptibility profile of 29 antibiotics was assessed by disc diffusion assay. The antibiogram of the strains demonstrated that all 20 strains are multiresistant to antibiotics, i.e., have resistance to more than two group of antibiotics. The presence of different antibiotic resistance genes was investigated by PCR. Based on the *bla*-encoding genes, OXA-aer and FOX were the most detected *β*-lactamases.

Biofilm formation of 18 strains, of the genus *Acinetobacter, Aeromonas, Klebsiella*, *Pseudomonas* and *Shewanella*, was assessed in microtiter plates and quantified using crystal violet (CV) staining. The overall results demonstrate that the strains showed different ability to form biofilms, even if some strains are of the same species, suggesting that adhesion and biofilm formation is a strain dependent process.

The activity of ciprofloxacin (CIP) on the control of *Aeromonas* spp. biofilms and the effect of imipenem (IPM) on *Acinetobacter* spp. AS0027A3a, *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1 and *S. putrefaciens* AS006C2 biofilms, revealed that antibiotics at their minimum inhibitory concentrations (MIC) and at 10× MIC were ineffective in total biofilm removal.

Evaluation of the ability of *A. salmonicida* AS006C3c1, *A. veronii* AS070GSP1, *Acinetobacter* spp. AS027A3a, *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1 and *S. putrefaciens* AS006C2 to survive under simulated human gastrointestinal tract conditions, revealed that two strains (*A. salmonicida* AS006C3c1 and *K. pneumoniae* AS027A2), are capable to survive for more than 24 hours under simulated human gastrointestinal tract conditions. Furthermore, all six strains were selected to determinate their ability of adhesion and invasion of human colon adenocarcinoma cells line (Caco-2 cells line). All the strains were able to attach to Caco-2 cells, among them *A. salmonicida* AS006C3c1 was the strain that presented the largest number of attached cells. When studying only Caco-2 cells invasion it was found that the strain with the highest invading capacity was *P. fluorescens* AS008A1.

The results collected in this work allowed to conclude that the presence of multiple antibiotic resistance and genetic determinants of resistance in these strains isolated from wild animal reveals a potential significant public health impact. Their ability to form biofilms increases the potential to resist antimicrobial treatments and therefore persist in the colonizing ecosystem.

RESUMO

Os antibióticos foram originalmente desenvolvidos para o tratamento de doenças infeciosas. No entanto, a resistência aos antibióticos tem vindo a dificultar globalmente o tratamento das doenças infeciosas. Hoje em dia, tem-se verificado um aumento do número de agentes patogênicos resistentes, alguns dos quais resistentes a múltiplas classes de antibióticos, com implicações tão para os animais como para o homem. Nos últimos anos, tem sido reconhecida a importância dos animais selvagens na transmissão de doenças infeciosas. As zoonoses representam um grande problema de saúde pública, o que levou a um aumento da vigilância na prevenção e controlo de doenças envolvendo o homem e animais. As bactérias podem desenvolver vários mecanismos de resistência aos antibióticos, tais como: alteração do local alvo do antibiótico, modificação e degradação enzimática, prevenção do acesso do antibiótico ao alvo. Outro fator importante que contribui significativamente para o aumento da resistência é a capacidade dos microrganismos para desenvolverem biofilmes. Os biofilmes são sistemas dinâmicos e complexos, com caraterísticas tanto de organismos multicelulares primordiais como de ecossistemas multifacetados. Considerando o problema de saúde pública causado com o aumento de bactérias multirresistentes no ambiente, é crucial conhecer a importância dos animais selvagens como reservatórios de bactérias multirresistentes e sua capacidade de formar biofilmes e invadirem às células humanas.

O principal objetivo deste estudo foi fornecer informação sobre a presença e o comportamento de bactérias resistentes em animais selvagens selecionados no Norte de Portugal. Algumas bactérias isoladas foram selecionadas para identificação e avaliou-se o seu perfil de susceptibilidade no estado planctónico e séssil.

Foi avaliada a capacidade de algumas estirpes sobreviverem sob condições do trato gastrointestinal humano. Além disso foi determinada a capacidade de adesão e invasão à linha celular adenocarcinoma do cólon humano (Caco-2), e a capacidade de produção de factores de virulência: *N*-acil homoserina lactonas (AHLs), sideróforos, proteases e gelatinases.

A identificação de 20 estirpes foi realizada por análise fenotípica e sequenciação dos genes 16s rDNA e *gyr*B. Foram identificadas 16 estirpes como pertencentes ao género *Aeromonas*. Com base nos alinhamentos das sequências de *gyr*B observou-se que as estirpes se agrupavam em 4 grupos filogenéticos com 9 sequências diferentes de *gyr*B: *Aeromonas salmonicida, A. eucrenophila, A. bestiarum* e *A. veronii*. As restantes estirpes pertencem aos géneros *Acinetobacter*, *Pseudomonas*, *Klebsiella* e *Shewanella*. O estudo do

perfil de susceptibilidade a 29 antibióticos foi avaliado por meio de ensaio de difusão do disco. O antibiograma das estirpes demonstrou que todas eram multirresistentes, isto é, apresentam resistência a mais do que dois grupos de antibióticos. A presença de *β*-lactamases foi avaliada por PCR, tendo sido as enzimas OXA-aer e FOX as mais detetadas.

A formação de biofilmes de 18 estirpes, dos géneros *Acinetobacter*, *Aeromonas*, *Klebsiella*, *Pseudomonas* e *Shewanella*, foi avaliada em placas de microtitulação e quantificada por coloração com cristal de violeta (CV). Os resultados globais demonstram que as estirpes apresentaram diferentes habilidades para formar biofilme, mesmo as estirpes pertencentes à mesma espécie. Estes dados sugerem que a adesão e formação de biofilme é um processo dependente da estirpe.

Foi ainda avaliada a atividade da ciprofloxacina (CIP) no controlo da formação de biofilmes. Além disso, o efeito do imipenemo (IPM) também foi testado em quatro estirpes (*Acinetobacter* spp. AS0027A3a, *P. fluorescens* AS008A1, *K. pneumoniae* AS027A2, *S. putrefaciens* AS006C2). Observou-se que a concentração mínima inibitória (MIC) e 10× MIC foram ineficazes na remoção total do biofilme.

A avaliação da capacidade das estirpes *A. salmonicida* AS006C3c1, *A. veronii* AS070GSP1, *Acinetobacter* spp. AS0027A3a, *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1, *S. putrefaciens* AS006C2 para sobreviver sob condições simuladas do trato gastrointestinal humano, revelou que duas estirpes (*A. salmonicida* AS006C3c1 e *K. pneumoniae* AS027A2) são capazes de sobreviver por mais de 24 horas sob condições simuladas do trato gastrointestinal humano. Além disso, as seis estirpes foram selecionadas para determinar sua capacidade de adesão e invasão da linha celular do adenocarcinoma do cólon humano (Caco-2). Todas as estirpes foram capazes de aderir às células Caco-2, entre elas, *A. salmonicida* AS006C3c1 foi a que apresentou o maior número de células aderidas. Ao estudar apenas a invasão das células Caco-2, observou-se que *P. fluorescens* AS008A1 era a que apresentava maior capacidade de invasão.

Os resultados deste trabalho permitiram concluir, que a presença de determinantes genéticos de resistência em bactérias isoladas de animais selvagens revelam uma grande importância para a saúde pública. A capacidade de formação de biofilmes aumenta o potencial dos microrganismos para resistir aos tratamentos antimicrobianos e, portanto, a sua persistência no ecossistema colonizado.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

Abbreviations

AACs	Adenylyl transferase
ABC	ATP binding cassette family
AHL	*N*-acyl Homoserine Lactones
AMEs	Aminoglycoside modifying enzymes
ANTs	Nucleotidyltransferase
APHs	Phosphoryl frases
ATP	Adenosine triphosphate
AVMA	American Veterinary Medical Association
BHI	Brain Heart Infusion
BR	Percentage of biofilm removal (%)
Caco-2	Human colon adenocarcinoma cells line
CAS	Chrome azurol S
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
DMEM	Dulbecco's Modified Eagle's medium
DMT	Drug/metabolite transporter superfamily
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substances
ESBL	Extended-spectrum- β-lactamases
EU	European Union
F	Fertility factor
FAO	Food and Agriculture Organization of the Unite Nation
FCS	Fetal calf serum
HGT	Horizontal gene transfer
MATE	Multidrug and toxic compounds extrusion family
MBLs	Metallo β-lactamases
MDR	Multiresistant
MHA	Muller-Hinton agar
MHB	Muller-Hinton broth
MIC	Minimal Inhibitory Concentration
MF	Major facilitator superfamily
OD	Optical density
OIE	World Organization for Animal Health
PBPs	Penicillin-binding proteins
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PS	Polystyrene

qnr	Quinolone resistance gene
QS	Quorum Sensing
RND	Resistance-nodulation-division family
rpm	Rotation per minute
rRNA	Ribosomal Ribonucleic acid
SMR	Small multidrug resistance family
SPSS	Statistical Package for the Social Sciences
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TSA	Trypticase Soy agar
TSB	Trypticase Soy broth
WCS	Wildlife Conservation Society
WHO	World Health Organization

Indexes

$^-$	Electron donor parameter of the Lewis acid-base component (γ^{AB})
$^+$	Electron acceptor parameter of the Lewis acid-base component (γ^{AB})
l	Diameter of bacterial growth inhibition
2	Total diameter
AB	Lewis acid-base component
LW	Lifshitz-van der Waals component
s	Surface
sws	Interaction between two entities of a given surface, when immersed in water
Tot	Total
w	Water

Greek letters

γ	Surface free energy (mJ/m2)
ΔG	Free energy of interaction (mJ/m2)
Θ	Contact angle (º)
λ	Wavelength (nm)

CHAPTER 1. WORK OUTLINE

1.1 BACKGROUND

Resistance to antibiotic was observed in pathogenic bacteria soon after the introduction of these drugs into human and veterinary medicine, around sixty years ago, leading to difficulties in the management of infectious diseases. At present, antibiotic resistance is recognized as an important problem to human and animal health (Livermore, 2009, Woodford *et al.*, 2011). Antibiotic resistance concern has been focusing mainly in human clinical purposes, despite the recognized importance of antimicrobial resistant bacteria spread between animals and humans (Duranand Marshall, 2005, Webster, 2009).

Through movement and trade of animals and food, bacteria and their resistance determinants spread through the environment and animals, up to the food chain to humans. Resistant bacteria from animal sources can spread by direct contact between humans and animals, including farm and wild animals and pets, by ingestion of meat and eggs, and by the fecal waste of animals discharged into environment (into manure, soil, water streams). However, few reports describe the prevalence of resistance mechanisms in pathogens recovered from animals (Raymond *et al.*, 2006, Linand Davies, 2007, Chee-Sanford *et al.*, 2009, Rodríguez *et al.*, 2009, Simpson *et al.*, 2009).

Wild migrating animals may also play a role in spreading antimicrobial resistance through the environment, animals and humans. *Escherichia coli*, *Aeromonas*, *Enterococcus* and *Campylobacter* with antibiotic resistances have been found in wild animals and migrating birds (Middletonand Ambrose, 2005, Waldenstrom *et al.*, 2005, Bonnedahl *et al.*, 2009, Dias *et al.*, 2014). The importance of wildlife as reservoirs of antimicrobial determinants is largely unknown but may reflect the transmission between farm animals and wild ones (Kruse *et al.*, 2004).

Beyond the dissemination of bacterial resistance through different routes, horizontal gene transfer is the key mechanism behind the wide dissemination of antibiotic resistance genes. The spread of resistance determinants is possible by the insertion of those genes within mobile genetic elements like plasmids, transposons and integrons. Recently, extended-spectrum β-lactamases such as CTX-M (an important reason of β-lactam therapy failure and whose emergence is associated with the use of extended-spectrum cephalosporins in human medicine), have been found in animals, aquatic systems and are disseminated worldwide (Henriques *et al.*, 2006, Tian *et al.*, 2009, Tacão *et al.*, 2012).

Bacterial adhesion to surfaces and biofilm formation is a significant phase in the pathogenicity of microorganisms (Borges *et al.*, 2012). In addition, biofilm establishment on abiotic or biotic surfaces reduce the efficacy of antibiotic, protecting bacterial cells from host defenses mechanisms and increases bacterial cell-cell communication, or quorum sensing (QS), resulting in the expression of virulence factors (Igbinosa *et al.*, 2015).

1.2 RESEARCH OBJECTIVES

In last year's antibiotic resistant bacteria of animal origin and its impact on human health have drawn much attention worldwide. Because most studies focus on domestic or farm animals and wild animals can act as forgotten reservoirs of antimicrobial resistant pathogens this project aimed at evaluating the presence of antimicrobial resistance in bacteria recovered from wild animals in the north of Portugal, and to investigate the genetic background aspects associated to the expression of antibiotic resistance.

The study started by collecting bacteria from wild animals entering the *Centre for Reception, Recovery and Treatment of Wild Animal* (CRATAS) of the University of Trás-os-Montes and Alto Douro. Fecal samples from different wild animal species (birds, reptiles and mammals) were collected aseptically, directly from the animal's rectum or cloaca, or from freshly voided faecal material.

Strains identification was done by phenotypic analysis, and 16s rDNA and *gyr*B sequencing. Secondly, the bacterial antimicrobial susceptibility profile was assessed by disc diffusion assay and by microdilution method to determine the minimal inhibitory concentration (MIC).

On basis of the antimicrobial susceptibility profile and characteristics of the strains, they were selected for further molecular screening by PCR. Emphasis was given to the detection of extended-spectrum *β*-lactamases (ESBLs) and carbapenemases. Many of these determinants are considered emergent resistant genes, and confer resistance to important clinically group of antibiotics in both human and veterinary medicine (Mendonça *et al.*, 2007, Mendonça *et al.*, 2008). The genes encoding these enzymes often coexist with other antibiotic resistance determinants and can also be associated with transposons/integrons increasing the potential enrichment of resistant strains to multiple antibiotic as well as dissemination of the resistance determinants among bacterial species (Li *et al.*, 2007).

Another aim of this study was to investigate the physicochemical surface properties of the strains and their ability to adhere to polystyrene (PS) and to form biofilms.

In addition, control of biofilms formed by selected bacteria with two antibiotics (ciprofloxacin - CIP and imipenem - IPM) was tested in 96-well PS microtiter plates. Twenty-four hours after exposure the biofilms were analyzed in terms of biomass reduction. Moreover, the mechanisms of quorum sensing (QS), an important process of intercellular communication, that is associated with different steps of biofilm formation and differentiation, was investigated based on the modulation of *N*-acyl homoserine lactones (AHLs) activity. To achieve these results, a qualitative agar diffusion assay based on the violaceum pigment by the AHL biosensor strain *Chromobacterium violaceum* CV026 was used.

As pathology caused by microorganisms involves several virulence factors, such as the ability to produce toxins, adhesion and invasion, another objective of this study was to evaluate the ability of selected bacteria from these isolated from the wild animals to attach and internalize in the human colon adenocarcinoma cells line Caco-2.

1.3 THESIS ORGANIZATION

The thesis is organized in nine chapters. In the first chapter the context and motivations for the development of this study are described. The justifications for the research purposes and the main objectives are also presented in this chapter.

Chapter 2 provides a brief review on the literature in relation to the work presented in the thesis, with special focus on antibiotic resistance. Antibiotics have long been considered the “magic bullet” that would end infectious disease. Although they have improved the health of countless numbers of humans, many antibiotics have also been losing their effectiveness in the treatment of infections. Gradually it has been recognized that this problem is not exclusive of humans, affecting also the animal’s health and the environment. Furthermore, animals may contribute to the spread of antibiotic resistance. The concept of “One Health” is an emerging integrated approach englobing the environment, animal and human health. Also, the problematic of antibiotic resistance on planktonic bacteria and biofilms, and their mechanisms the resistance are focused in this chapter.

Chapter 3 describes the collection of strains obtained from wild animals and their identification and characterization. The material and methods used to complete the experimental work of this thesis are also presented.

Chapter 4 reports the isolation from wild animals and identification of *Aeromonas* spp., an emergent human pathogen, their antibiotic resistance phenotype and its correlation with

the presence of β-lactamases genes (*bla*$_{CphA}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{OXA}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{MOX}$ and *bla*$_{FOX}$).

Chapter 5 focuses the characterization of the adhesion and biofilm formation abilities of 14 distinct *Aeromonas* spp. strains (eight *A. salmonicida*, three *A. eucrenophila*, two *A. bestiarum* and one *A. veronii*). Biofilm formation was studies because it significantly increases antibiotic resistance leading to treatment failure.

Chapter 6 reports the determination of the antibiotic resistance patterns and biofilm production ability of four strains (*Acinetobacter* spp. AS027A3a, *Klebsiella pneumoniae* AS027A2, *Pseudomonas fluorescens* AS008A1 and *Shewanella putrefaciens* AS006C2) isolated from different species of wild animals, and resistant to imipenem, an important clinical antibiotic.

Chapter 7 describes the studies developed to determine the ability of six strains (*A. veronii* AS070GSP1, *A. salmonicida* AS006C3c1, *Acinetobacter* spp. AS027A3a, *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1 and *S. putrefaciens* AS006C2 to survive under simulated human gastrointestinal conditions. Besides, the planktonic growth curves determined for two strains (*A. salmonicida* AS006C3c1 and *K. pneumoniae* AS027A2) that showed ability to survive under simulated human gastrointestinal conditions are presented.

In Chapter 8 the attached and invasion assay of six strains selected from the previous chapters (*A. veronii* AS070GSP1, *A. salmonicida* AS006C3c1, *Acinetobacter* spp. AS027A3a, *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1 and *S. putrefaciens* AS006C2) were assessed on a human colon adenocarcinoma cells line Caco-2. In this chapter, was also evaluated the six strains capacity to produce *N*-acyl homoserine lactones (AHL), siderophores, proteases and gelatinases, factors associated with their virulence.

In Chapter 9 the main conclusions of this thesis are presented, together with perspectives for further research.

CHAPTER 2. LITERATURE REVIEW

This study focuses on multiresistant bacteria isolated from different wild animals. Many factors have been described to the ascending levels of bacterial cell resistance to antibiotics used in both human and animal medicine and have led to the emergence of multiresistant strains. Resistance to antibiotics was observed in pathogenic bacterial strains soon after the introduction of these drugs into human and veterinary purposes, leading to difficulties in the management of infectious diseases. At present, antimicrobial resistance is recognized as a huge problem in human, animal, and environmental health. Through movement and trade of animals and food, bacteria and their resistance determinants can disseminate among animals, up in the food chain, in the environment and spread to humans.

2.1 ANTIBIOTIC RESISTANCE: AN EMERGING PROBLEM

An antibiotic is a chemical substance that can be produced by fungi, bacteria, and other organisms or by a synthetic substance used to kill (bactericidal) or inhibit the growth of other microorganisms (bacteriostatic) (Galerunti, 1994). In general, the term antibiotic refers to a small molecule and their synthetic and semisynthetic derivatives, used in the treatment of infectious diseases caused by pathogenic bacteria. On the other hand, an antimicrobial agent is a global term used to mention a group of chemical compounds that comprises antibacterial, antifungals, antiprotozoals and antiviral agents (Levy, 1997, Oidtmann *et al.*, 2013).

Since their introduction in the 1930`s, the antibiotic agents have been considered one of the most important discover in human medicine advances (Figure 2.1), reducing morbidity and mortality from infectious diseases (Cosgrove, 2006). Antibiotics are used for the treatment of a wide range of microbial infectious diseases and are important in other medical treatments, namely organ transplantation, chemotherapy, intensive care, which could not be implemented effectively without the available effective antibiotics (Marti *et al.*, 2014).

One of the most important and sustained driving forces for antibiotics development over the last years has been antibiotic resistant strains (Marti *et al.*, 2014). The term resistance has been used to refer the temporary or permanent ability of a microorganism and its progeny to persist viable and/or multiply under conditions that would destroy or inhibit the growth of other microorganisms (Džidic *et al.,* 2008). Since microorganisms are capable to rapidly adjust to new environmental conditions such as the presence of antibiotic, resistance may increase within the increasing exposure to antibiotic (Daviesand Davies, 2010).

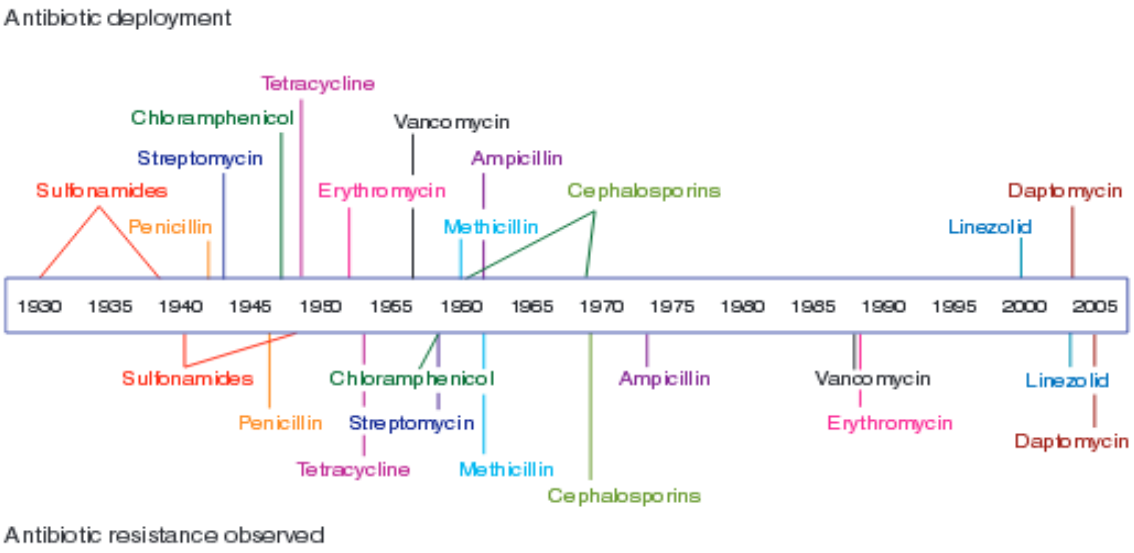

Figure 2.1 Timeline of antibiotic deployment and the evolution of antibiotic resistance. Adapted from Clatworthy *et al.* (2007).

The fast emergence of resistant bacteria was the first issue derived from the widespread and uncontrolled application of antibiotics (Beovic, 2006). Worldwide, there is a great concern about the problematic of resistance to antibiotics administrated in both humans and animals. The excessive use of antibiotics represents an important factor for the selection of resistant bacteria, not only among pathogenic bacteria but also among commensal strains (French, 2010). Strains that showed resistance to the antibiotics used in the medicine throughout the world has been detected and in many of the cases theses strains are resistant to multiple antibiotics (Cohen, 2000).

Although multiresistance has been a growing problem since the antibiotic's development, in last years occurred an increase in the number, diversity and range of resistant microorganisms (Wright, 2007, Daviesand Davies, 2010, Munitaand Arias, 2016). After the introduction of the first antibiotic, which was the harbinger of a new infectious disease treatment, some infectious microorganisms such as multiresistant *Acinetobacter baumannii* and *Klebsiella pneumoniae* have increased (Giamarellos-Bourboulis *et al.*, 2006, McGowan, 2006). According to information from the European Centre for Disease Prevention and Control (ECDC), and the European Medicines Agency (EMA), every year around 25 000 European inhabitants die from infectious diseases caused by multiresistant bacteria. The emergence of multiresistant bacteria is today an urgent and global problem in the human, animal and environmental health (Marti *et al.*, 2014).

This increasing resistance involves many common human pathogenic bacteria, namely *Enterococcus faecium*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and other Enterobacteriaceae (Boucher *et al.*, 2009, Carlet *et al.*, 2011).

The phenomenon of multiresistant bacteria is a consequence of the genetic plasticity of microorganisms. Multiresistance can be the result of multiple and complex mechanisms namely: (i) exclusion of the antibiotic by the outer membrane; (ii) intracellular modification and/or deactivation of the antibiotic; (iii) reduction in sensitivity of the cellular target; (iv) extrusive from the cell and (v) intracellular sequestration (Taylor *et al.*, 2011). These mechanisms can be acquired by bacterial mutation and selection or by acquisition, from other bacteria, the genetic information that encodes resistance genes (Marti *et al.*, 2014). Nowadays, medical specialists are warning of a coming back to the pre-antibiotic time. A recent database list the existence of more than 20 000 potential resistance genes (r genes) of approximately 400 different types, predicted in the main form of available bacterial genome sequences (Liuand Pop, 2009).

Multiresistant bacteria are present in every country. Patients with infections caused by multiresistant bacteria are at an increased risk of worse clinical outcomes and, therefore, demand more health-care resources (Llorand Bjerrum, 2014). In particular, multidrug resistance is now common in familiar pathogens such as *Staphylococcus aureus* and *Mycobacterium tuberculosis*, as well as emerging pathogens such as *Acinetobacter baumannii* (Wright, 2007). Resistance in *Klebsiella pneumoniae*, a common intestinal bacterium that can cause life-threatening infections, to a last resort treatment (carbapenem antibiotics) has spread to all regions of the world. Resistance to fluoroquinolone antibiotics in *E. coli*, one of the most widely used therapeuthic for the treatment of urinary tract infections is very widespread. There are countries in many parts of the world where this treatment is now ineffective in more than half of the patients (Wright, 2007, Pitout *et al.*, 2015).

Multiresistance bacteria spreading is aggravated due to the excessive use of antibiotics in both humans and animals, as consequence of the growing number of invasive medical procedures and the increase in the number of immunocompromised patients with chronic debilitating diseases (Turnidgeand Christiansen, 2005, Enne, 2010). Nowadays, there are several policies governing the use of antibiotics, in attempts to control the progressive spread of multiresistant strains (da Costa *et al.*, 2013).

Most of the pathogenic multiresistant strains, in the past, were isolated from healthcare facilities, in which the antibiotics used were prevalent, however, nowadays these bacteria can be routinely isolated from the community and environment samples. However, and increasing the concern, multiresistant strains are also associated with increased expression of virulence factors (Robinson *et al.*, 2005, Wellington *et al.*, 2013).

2.2 ENVIRONMENTAL RESERVOIR OF ANTIBIOTIC RESISTANCE

In addition to the consequences for public health, equally serious environmental and epidemiological effects result from antibiotic resistance (Rice, 2007), and for this reason studies approaching on antibiotic resistance should not be confined to clinical-associated bacteria. It was evident, soon after introducing the first antibiotic for human therapy, that microorganisms were capable to acquire multiresistant mechanisms, not just as the consequence of mutations, but by acquiring genes conferring resistance to antibiotics (Abrahamand Chain, 1988). Since those genes were not found previously in the human bacterial pathogens, the only suitable theory for its origin is the existence of an environmental resistome (Gardner *et al.*, 1969, Wright, 2007).

The presence of multiresistant microorganisms in unimpaired natural habitat was detected in ancient microbial community from 10 000 to 3 million years, in marine sea sediments, permafrost, pure high mountain lakes, soil depth (170 - 259 m) and in the microbiota of caves (Kozhevin *et al.*, 2013). Multiresistant microorganisms existed before the beginning of the antibiotic's use, being a trait that is inherent to microorganisms in the environment (Martinez, 2012, Kozhevin *et al.*, 2013). Recent research suggests that multiresistant bacteria occur in the environment has an ancient origin, which is not interrelated with anthropogenic activities (Tamae *et al.*, 2008, Olivares *et al.*, 2013). Thence, natural environments can be important, as they can act as reservoirs for antibiotic resistant bacteria and antibiotic resistant genes (Martinez, 2008, Forsberg *et al.*, 2012). Such natural environments include glaciers (Segawa *et al.*, 2013), soils (Riesenfeld *et al.*, 2004, Allen *et al.*, 2009), and wild animals (Foti *et al.*, 2009, Miller *et al.*, 2009, Martiny *et al.*, 2011).

It is reported that the emergence of multiresistant bacteria in human health is accompanied by the frequent detection of such bacteria in remote areas without human action (Sjolund *et al.*, 2008). Additionally, the antibiotic genes found in environmental bacteria have also been found in clinical pathogens (Forsberg *et al.*, 2012). Therefore, interactions between humans, domestic and wild animals represent an interface for the dissemination of

multiresistant bacteria (Figure 2.2). The incidence of resistant strains in wildlife seems to correlate with the degree of association with human activities (Skurnik *et al.*, 2006). The anthropogenic activity that interferes in the wildlife are generally including into two related categories: human invasion into wildlife habitat, and increasing interest in wildlife species. As the human population continues to grow and invade previously remote areas, there are increasing interactions between human and wildlife (Rhyanand Spraker, 2010).

The misuse of antibiotics by man is perhaps not the only selective pressure for antibiotic resistance in the environment: compounds and conditions that occur in the environmental bacteria may provide additional selection pressure. Indeed, most antimicrobial molecules are produced by strains of fungi and bacteria that occur naturally in all environments, including the soil (Martinand Liras, 1989). Most antimicrobial producing microorganisms can carry resistance genes to the antibiotics that themselves produce (Hopwood, 2007, Tahlan *et al.*, 2007), that frequently encode in the same gene cluster as the antibiotic biosynthesis pathway gene (Martinand Liras, 1989).

Antibiotics used in both urban and agricultural sources persist in aquatic environments and soil, and the selective pressure imposed by antibiotic can affect human's therapy (Thiele-Bruhn, 2003, Segura *et al.*, 2009). Agriculture manure and biological soils applied to land might contain both antibiotics and resistant bacteria. Therefore, the run-off from fertilized land or directly from the sewage can lead to contamination of surface water and consequently spread to human beings and animals through possible contact with soil, irrigation of crops, contact with water or with wildlife (Laxminarayan *et al.*, 2013).

Several works have demonstrated the presence of biologically active antimicrobial residues in human and animal waste and sewage (Kummerer, 2009). One example is when waste from sewage treatment plants originating from hospitals, but also manure, that contains both antibiotic and a variety of human and animal associated bacteria, are spread out on fields as fertilizers (Baquero *et al.*, 2008).

The environmental contamination by quinolones is an example from aquatic environment. Quinolones are poorly degraded and still active in the environment after release through human and animal urine (Poirel *et al.*, 2005). They are also used extensively in fish and shrimp farming in parts of the world. The quinolone resistance gene "*qnr*", is present in the chromosomes of waterborne bacteria, where it has a so far unknown function (Cattoir *et al.*, 2008).

Once present in the environment, other substances than the antibiotic itself could contribute for the presence and diffusion of antibiotic resistance. For instance, heavy metal

pollution can select for antibiotic resistance (Hernandez *et al.*, 1998), and stress conditions, as found in polluted environment, have the potential to increase recombination and horizontal gene transfer, a pathway that favors the dissemination of antibiotic resistance genes (Beaber *et al.*, 2004).

The high mobility of human population and the food production, as well the absence of environmental barriers between the different ecosystem niches raised the risk for transmission and dissemination of multiresistant bacteria worldwide (Acarand Rostel, 2001, Memish *et al.*, 2003, Silbergeld *et al.*, 2008). The spread of disease-causing bacteria can have serious impacts to the human and animal health. Consequently, understanding microorganism's dissemination on this interface is crucial for the long- term conservation of endangered species (Jones *et al.*, 2013). Same studies recognized that the origin and spread of antibiotic resistance is a very important and complex problem (Cantas *et al.*, 2013, Galán *et al.*, 2013, Gaze *et al.*, 2013).

Antibiotics are one of the most prescribed drugs. Since the development and the introduction of antibiotic in medicine, every year a wide range of tons of active antibiotics enters into the environment (Demainand Sanchez, 2009, Daviesand Davies, 2010). Around half of these antibiotics are used in human and veterinary medicine for treatment and/or prevention of infections, and the other half is used for agriculture (Aminovand Mackie, 2007, Landers *et al.*, 2012). With the overuse/misuse use of antibiotic in the modern medicine, multiresistant microorganisms and their resistant genes are released into our environment (Singer *et al.*, 2003, Turnidge, 2004). In human and veterinary medicine practices, the same conditions lead to the selection and spread of multiresistant bacteria (Silbergeld *et al.*, 2008). The majority of the antibiotics used for the treatment of several infectious diseases in animal health frequently are the same or similar to those used in human's medicine (Marshalland Levy, 2011). There are two major resistance pathways supporting the antibiotic resistance spread: transmission of inter bacteria harboring the resistance genes and/or the specific transmission of the concerned resistant genes. This process can occur between bacteria from different origin and ecosystems (Cantas *et al.*, 2013).

Multiresistant microorganisms and genetic determinants of resistance can enter into the environment through the discharge of untreated or incompletely treated wastewater from hospitals, application of activated sludge from wastewater treatment plants as fertilizer to agricultural soil, territories of agriculture farms or slaughterhouses; sewage overflows during heavy rainfall and run off of animal manure or faeces of pasture animals (Iversen *et al.*, 2002, Reinthaler *et al.*, 2003, Costa *et al.*, 2008, Chagas *et al.*, 2011, Wang *et al.*, 2012). Antibiotics

can remain chemically active after the wastewater treatment and be released into the environment (Lindberg *et al.*, 2004, Lindberg *et al.*, 2005, Watanabe *et al.*, 2010). When a microorganism enters into the environment, bacteria of different origins come into physical contact and the environment is considered to be the natural reservoir of resistance genes from those bacteria (Genthner *et al.*, 1988, Xu *et al.*, 2007). Beside the clinical consequence, equally serious ecological and epidemiological effects results from the emergence of spread of multiresistant bacteria (Rice, 2007).

The World Health Organization (WHO) in last years announced a group of strategies that, if implemented, should mitigate the emergence and dissemination of multiresistant bacteria (Leung *et al.*, 2011, Europeia, 2017). The same organization recently recognised antibiotic resistance as a serious global problem in terms of the public, environmental and animal health (Figure 2.2) (Leung *et al.*, 2011, Gibbs, 2014).

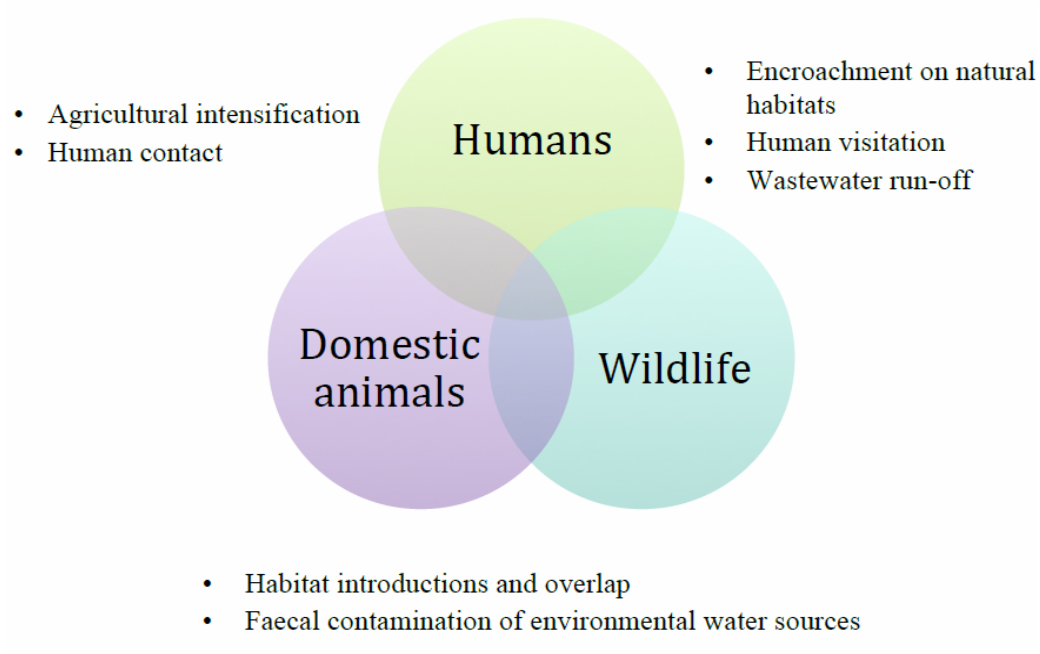

Figure 2.2 The human, domestic animal and wildlife interface. Anthropogenic impacts to natural environments and habitation overlap with terrestrial species provide increased opportunities for the dissemination of microorganism's wildlife. Adapted from Gormaz *et al.* (2014).

Several studies demonstrated that antibiotic-resistant strains are present in a wide range of ecological niche, including wild species that habit in remote ecosystems. Multiresistant strains from either human, animal or environment may cross link between each interface (Smith *et al.*, 2014).

Multiresistant bacteria were detected in wild animal, namely wild birds (birds of prey and gulls) and wild mammals (deer, foxes, rabbits and wolves) (Middletonand Ambrose, 2005). These studies suggest that multiresistant bacteria, once developed, is not limited to the ecological niche where it primarily emergent. The multiresistant bacteria isolated from human medicine are very similar to the multiresistant bacteria obtained from wild animals (Simões *et al.*, 2012, Gorski *et al.*, 2013). In general, wild animals could therefore be used as reservoirs of genetic determinants of antibiotic resistance and of multiresistant bacteria. Moreover, strains of *E. coli* are ubiquitous and, asymptomatically colonize the gut of most of all birds and mammals, and its spread in the environment is almost inevitable (Wirth *et al.*, 2006, Goldberg *et al.*, 2008, Rwego *et al.*, 2008). In the recent years, several works have identified the presence of extended-spectrum *β*-lactamases (ESBLs) in strains isolated from birds of prey (Bonnedahl *et al.*, 2009, Bonnedahl *et al.*, 2010, Guenther *et al.*, 2010, Smet *et al.*, 2010, Wallensten *et al.*, 2011).

About 61% of the several infectious diseases in humans are zoonotic (Cunningham, 2005). The WHO defined a zoonoses as "*a disease or infection caused by all types of agents (bacteria, parasites, fungi, viruses and unconventional agents) transmissible from vertebrate animals to humans and vice versa*" (http://www.who.int/topics/zoonoses/en/). Most of all zoonotic infectious diseases originated in wildlife, and their incidence in the last years has increased (Jones *et al.*, 2008). Wildlife animals can also be a reservoir for nonzoonotic diseases of livestock, potentially leading to human economic loses, as well as the loss of human dietary protein (Rhyanand Spraker, 2010).

2.3 ONE HEALTH: AN APPROACH TO CONTROL ANTIBIOTIC RESISTANCE

Anthropogenic activities and global changes have huge impact on the environment, perturbing the ecosystems, plant and animal demographics and behaviours. These alterations increase the cross-link between and among species, consequently increasing the pace on the emergence of infectious diseases (Anderson *et al.*, 2004, Gortazar *et al.*, 2014).

In the last years, there was a global concern associated to emerging infectious diseases. These worldwide emerging concern drew attention to the interconnection between humans and the etiological sources of their infectious diseases, which spurred the implementation of the global control of such infectious diseases by multidisciplinary teams (Gibbsand Gibbs, 2013). Pathogenic microorganisms can escape their natural wildlife reservoirs and infect humans and domestic animals upon cross-species diffusion (Lloyd-Smith *et al.*, 2009).

Occasionally, the same pathogenic agents that are newly recognized or newly evolved, or that have occurred previously, shows an intensification in incidence or expansion in geographical area, in vector range or host (Cutler *et al.*, 2010). The consequences of emerging pathogenic bacteria in newly infected species, in wild or domestic animals, or even in new geographical areas, can have dire repercussions on human health and welfare, namely through the disruption of ecosystem services or large agricultural economic losses (Anderson *et al.*, 2004, Cunningham, 2005).

Zoonotic infections can be transmitted from animals to humans in many ways: (i) the transition may occur through animal bites and scratches; (ii) zoonotic infections derived from animals can reach people through direct faecal route, contaminated animal food products, improper food handling, and inadequate cooking; (iii) formers and animal health workers are at increased risks of exposure to certain zoonotic pathogens ant they may catch zoonotic microorganisms; they could also carry zoonotic infectious diseases that can be spread to other humans in the community; (iv) vectors, frequently arthropods (mosquitoes, ticks, fleas and lice) can actively or passively transmit bacterial zoonotic infectious diseases to humans; (v) soil and water recourses, can be contaminated with manure contain zoonotic pathogens bugs and pool of resistance genes that are available for transmission of bacteria that cause human infectious diseases (Roeand Pillai, 2003, Schauss *et al.*, 2009, Rascalou *et al.*, 2012, Cantasand Suer, 2014).

Scientists and governmental associations worldwide, recognised that greater interdisciplinary collaboration is required to prevent and control zoonotic infectious diseases, and that such association should include not only physicians and veterinarians, but also environmentalists, wildlife specialists, anthropologists, economists and sociologists, among others (Gibbs, 2005).

The "One Health" concept seeks to understand interactions between humans, animals and the ecosystems in which they co-exist, and in return, the impact on human and environmental health (Mi *et al.*, 2016). "One Health" strives to generate far-reaching impacts on global health, welfare, food security and poverty alleviation thought interdisciplinary science and integrated control of infectious diseases. The "One Health" origin lies in comparative medicine, the idea that there is not barriers between humans, animals and the environment when it comes to infectious diseases and health (Gibbsand Gibbs, 2013).

In the 20th century, Calvin Achwabe and James Steele used for the first time the term "One World, One Medicine, One Health" to refer to this transdisciplinary work (Monath *et al.*, 2010). There are several definitions to "One Health" concept, but the common thread is

collaboration on a globally scale among transdisciplinary science to ensure the health of humans, domestic animals and the environment (Figure 2.3), including wildlife, in the industrialized and evolved world (Gibbsand Anderson, 2009, Okello *et al.*, 2011).

According to the One Health Initiative Taskforce Report from American Veterinary Medical Association (AVMA, 2008) "One Health" concept is the "*collaborative effort of multiple disciplines working locally, nationally and globally to attain optimal health for people, animals and our environment*". The European Union (EU) define "One Health" as the "*improvement of health and well-being through (i) the prevention of risks and mitigation of effects of crises that originate at the interface between humans, animals and their various environments, and (ii) promoting a cross-sectoral collaborative, "whole of society", approach to health hazards, as a systemic change of perspective in the management of risks*". This definition was adopted by the WHO, Food and Agriculture Organization of the Unite Nation (FAO) and World Organization for Animal Health (OIE) applied a more restricted definition, focusing on zoonotic infectious diseases.

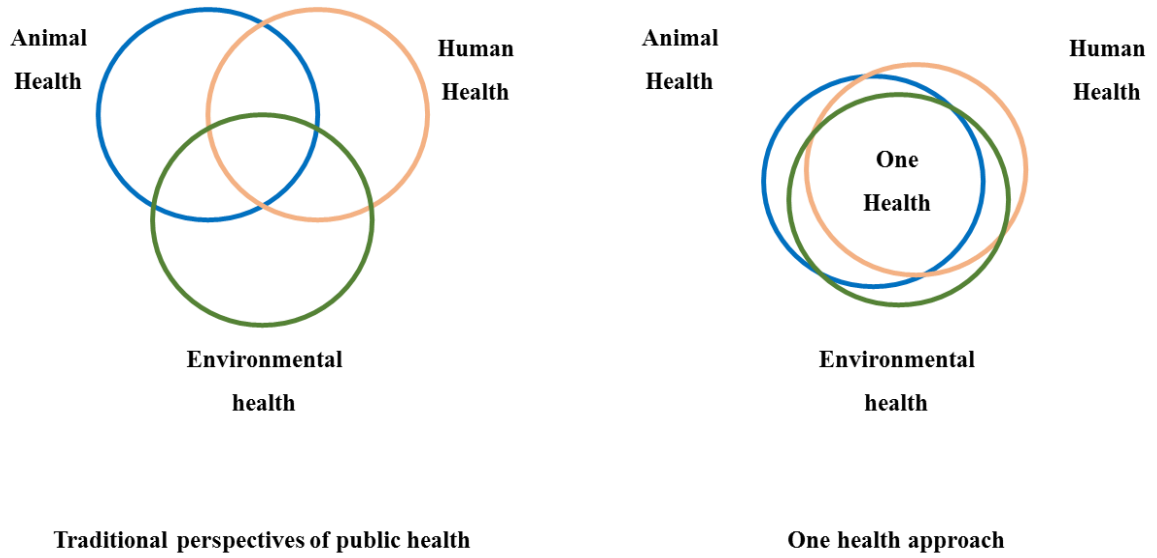

Figure 2.3 The health of humans, domestic animals, and the ecosystem are all connected. Evolution of "One Health" concept. Adapted from http://www.natureconservancy.ca/en/blog/canadian-federation-of-humane.html.

"One Health" approach is promoted to help tackle the increase multiresistant bacteria's dissemination, and same recommendations has been proposed: (i) to strengthen national multisectoral coordination for containment of antibiotic resistance; (ii) to strengthen national surveillance of antibiotic resistance; (iii) to promote national strategies for rational use of antibiotic and strengthen national surveillance of antibiotic consumption;

(iv) to strengthen infection control and surveillance of antibiotic resistance in health care settings; (v) to prevent and control the development and spread of antibiotic resistance in food-chain; (vi) to promote innovation and research on new molecules and technology; (vii) and to improve awareness, patient safety, and partnership (Queenan *et al.*, 2016).

2.4 MECHANISM OF ANTIBIOTIC RESISTANCE ACQUISITION AND DISSEMINATION

In response to the increased use of antibacterial compounds throughout the 20th century, bacteria have evolved ways to overcome the effect of antibiotics and thereby become resistant. Several factors have been attributed to the ascending level of bacterial resistance to antibiotics used in clinical settings and have led to the emergence of multiresistant strains. In the same way, antibiotic use in nonhuman health is another important reason for the emergence and spread of multiresistant microorganisms (Martinezand Baquero, 2002).

Microorganisms can be intrinsically resistant to antibiotics or can also acquire resistance to antibiotics. The intrinsic resistance to antibiotic actions is an ability that one bacterial species has as a result of inherent biology or functional characteristic. For example, *β*-lactam resistance in *Mycoplasma* spp. is due to this genus lack of cell wall, or vancomycin resistance in Enterobacteriaceae due to the outer membrane of Gram-negative bacteria (Blair *et al.*, 2015).

There are two main mechanisms that bacteria can use for developing antibiotic resistance, namely mutations in gene(s) often associated with the mechanism of action of the compound (point mutation, deletions and inversions), and the acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT) (Bennett, 2008, Fajardo *et al.*, 2008).

However, an in depth understanding of mutational resistance has until recently been unfeasible due to limited sequencing capabilities. With the advent of high-throughput sequencing platforms this situation is now changing, being possible to sequence bacterial genomes at a large scale and identify single nucleotide polymorphisms (SNPs) across sensitive and resistant strains (von Wintersdorff *et al.*, 2016). High frequency of mutator phenotypes among human pathogenic bacteria was described, for example in *Salmonella enterica*, *P. aeruginosa* and *E. coli* isolated from clinical samples and other natural environmental strains (LeClerc *et al.*, 1996, Oliver *et al.*, 2000).

Acquisition of foreign DNA material through HGT is one of the most important drivers of bacterial evolution and is frequently responsible for the development of antimicrobial resistance (von Wintersdorff *et al.*, 2016). Classically, bacteria acquire external genetic material through three main strategies (Figure 2.4): transformation (incorporation of naked DNA), transduction (phage mediated) and, conjugation (bacterial "sex") (von Wintersdorff *et al.*, 2016).

Once acquired, new resistance genes are not easily lost (Hurdle *et al.*, 2005, Tenover, 2006, Alekshunand Levy, 2007, Džidic *et al.*, 2008, Hawkey, 2008). HGT is responsible for the diffusion of multiresistant from commensal and environmental bacteria to pathogenic ones, and as has been reported as clinically important antibiotic resistance genes (von Wintersdorff *et al.*, 2016).

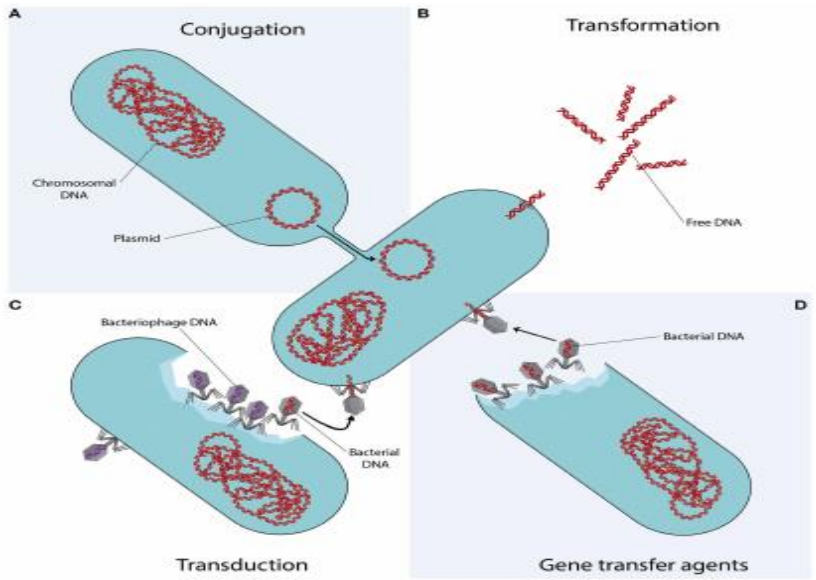

Figure 2.4 Mechanisms of horizontal gene transfer. (A) Conjugation is a process requiring cell to cell. (B) Transformation is the uptake, integration, and functional expression of naked fragments of extracellular DNA. (C, D) Transduction, bacteriophages may transfer bacterial DNA from a previously infected donor cell to the recipient cell. Adapted from von Wintersdorff *et al.* (2016).

Transformation is a process in which bacteria take a small exogenous DNA from the environment (Dubnau, 1999). In microorganisms the uptake of exogenous DNA is a complex process that requires expression of the genes involved in the assembly of type IV pili and type III secretion systems (Chenand Dubnau, 2004). This process are not the same in Gram-negative and Gram-positive bacteria due to the physiology of the cell wall. In

Gram-negative strains, and due to the presence of an extra membrane in cell wall, the presence of a more complex channel to DNA uptake is required (Chenand Dubnau, 2004).

The term transduction refers to the process of gene transfer in which a bacteriophage (phages) transfer DNA from one strain to other. Once inside the host cell, the phage can integrate its genome into the host genome and take over the cell machinery to synthesize new copies of its genome as well as all the proteins required for packing and structure (Hoffman, 2001, Chenand Dubnau, 2004).

Transformation is perhaps the simplest type of HGT, but only a handful of clinically relevant bacterial species are able to "naturally" incorporate naked DNA to develop resistance. Emergence of resistance in the hospital environment often involves conjugation, a very efficient method of gene transfer that involves cell-to-cell contact and is likely to occur at high rates in the gastrointestinal tract of humans (Hoffman, 2001, Chenand Dubnau, 2004).

Bacterial conjugation is the only mechanism of gene transfer that needs direct contact between cells. This exchange is unidirectional, i.e. one bacterial cells are the donor and the other is the recipient cell. In this way, gene is transferred laterally amongst existing bacteria as opposed to vertical gene transfer in which genes are passed on to offspring. The donor cell carries a special type of plasmid or fertility factor (F) which promotes gene transfer (Chenand Dubnau, 2004).

Not surprisingly, bacteria have evolved sophisticated mechanisms of drug resistance to avoid killing by antimicrobial molecules, namely through the production of enzymes that degrade or inactive the antibiotic, by the production of an unconventional metabolic pathway that bypass the action of the antibiotic, or by the expression of efflux pumps that prevent the antibiotic from reaching its intracellular target (Figure 2.5) (Spratt, 1994, Webberand Piddock, 2003, Woodfordand Ellington, 2007).

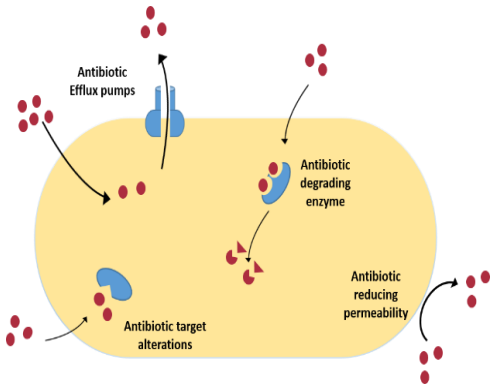

Figure 2.5 Mechanism of antibiotic resistance. Adapted from Borges *et al.* (2016).

2.4.1 Prevention of access to target

Outer membrane permeability and antibiotic resistance

The cell membranes of Gram-negative and -positive bacteria are structurally different (Blair *et al.*, 2015). Many of the infectious diseases are caused by Gram-negative bacteria, which have an additional membrane layer called outer membrane (OM). The OM is a largely asymmetric bilayer composed of glycerol phospholipids and lipopolysaccharides (LPs) (Giedraitienė *et al.*, 2011). Bilayer works as a barrier for protection against toxic molecules, including antibiotics, whose many antibiotics applicate in the human therapy possess a target site of action inside the cell cytoplasm (e.g. inhibitors of protein, DNA and RNA synthesis) or inside the cytoplasmic membrane (e.g. β-lactam antibiotics) (Blair *et al.*, 2015).

The cell walls in Gram-positive bacteria are mostly constituted by peptidoglycan, which accounts for 50% of the weight of the wall (Koch, 2000). Resistance of Gram-positive strains is usually related to mechanisms involving destruction or inactivation, changes in the target site, or active efflux (Russell, 1998). Exclusion by the cell wall is not usually responsible for resistance. Among the larger-sized antibiotics, glycopeptides (1240 Da), rifampicin
(823 Da) and fusidic acid (516 Da) are notable to penetrate the wall to interact with the cytoplasmic membrane (Friedrich *et al.*, 2000).

In Gram-negative bacteria, the influx is controlled essentially by porins, which are water-filled open channels that extent the OM and permit the passive penetration of hydrophilic molecules. Several different types of porins have been described and characterized in these microorganisms. Porins channels are classified according to their functional structure (monomeric or trimeric), their activity (specific channel or selective pore) and their regulation and expression. Previous studies showed that in main Enterobacteriaceae the major outer-membrane porins are thought function as a non-specific channels (Blair *et al.*, 2015), for example *E. coli* strains can produce three major trimeric porins OmpF, OmpC and PhoE (Nikaido, 2003) or through several *P. aeruginosa* porins like OprB and OprD (Wylieand Worobec, 1994, Ochs *et al.*, 1999).

Antibiotics undertake two pathways to penetrate the outer membrane targeting the cytoplasmic membrane, the lipid-mediated pathway and general porins protein diffusion.

Some antibiotics use both ways to penetrate the cell, namely quinolones and tetracycline. Hydrophobic antibiotics penetrate the Gram-negative cell outer membrane *via* lipid-mediated pathway whereas the hydrophilic antibiotics use the proteins porin to enter the bacterial cell (Delcour, 2009). The antibiotic gentamicin, kanamycin, rifamycin, fusidic acid and cationic peptides are hydrophobic and can enter to the cell through the outer membrane bilayer (Nikaido, 2003).

Active efflux pump mediated antibiotic resistance

Antibiotic resistance can be explained in biochemical terms as the inability of an antimicrobial agent to reach its bacterial target at an adequate concentration for inhibiting the target activity. Within this scope one way of acquiring resistance is diminishing the antibiotic concentration inside the bacterial cell. Efflux pump is considered one major mechanism by which microorganisms can expel antibiotic outside the cell, since the active export of antibiotic from the bacterial cell is an extremely effective mechanism resistance (Blanco *et al.*, 2016). These transport proteins are found in all microorganisms, and the genes that encode efflux pump can be localized in the bacterial chromosome or in a mobile genetic element, such as plasmids (Piddock, 2006). In the last years, the identification and characterization of efflux pumps have receive more attention due to the actual multiresistant problem. The efflux pumps can be specific for subtract, or can transport a wide range of unrelated substance, and are known as multiresistant (MDR) efflux pumps (Blair *et al.*, 2015).

Efflux pumps systems, capable of exporting antibiotic, are generally classified into five major families: (i) the major facilitador (MF) superfamily; (ii) the ATP (adenosine triphosphate)- binding cassette (ABC) family; (iii) the resistance-nodulation-division (RND) family; (iv) the small multidrug resistance (SMR) family (a member of the much larger drug/metabolite transporter (DMT) superfamily); and (v) the multidrug and toxic compounds extrusion (MATE) family (Van Bambeke *et al.*, 2000).

Efflux pump systems have been referred in a large number of clinically important multiresistant bacteria, including the Gram-negative *E. coli* (AcrAB-TolC, AcrEF-TolC, EmrB, EmrD) (Poole, 2000), *P. aeruginosa* (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) (Poole, 2000) and the Gram-positive *Streptococcus pneumoniae* (PmrA) (Gill *et al.*, 1999) and *S. aureus* (NorA) (Kaatzand Seo, 1995). All of these systems efflux fluoroquinolones pumps and RND pumps (CmeB, AcrB and the Mex pumps) also export multiple antibiotics (Webberand Piddock, 2003). Single or multiresistant *E. coli* is is part

attributed to the occurrence of efflux pumps in addition to other resistant mechanisms. More than 37 efflux pumps, belonging to distinct families were described in the genome of *E. coli* strains. In strains of *P. aeruginosa*, twelve types of RND types efflux pumps have been found as responsible for resistance to antibiotics, detergents, chemical compounds, molecules, dyes and antiseptics, for example MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexGHI-OprD and MexXY efflux pumps. The MexAB-OprM efflux systems are responsible for resistance to a wide range of antibiotics namely *β*-lactams, tetracycline, trimethoprim, chloramphenicol, with intrinsic resistance toward fluoroquinolones (Askoura *et al.*, 2011).

2.4.2 Enzymatic inactivation or modification

Enzyme-catalyzed inactivation is the main mechanism that bacteria use to escape the action of the antibiotic. A diverse range of enzymes have been described as capable to inactivate and/or modify antibiotics of several classes, namely *β*-lactamases and aminoglycoside-modifying enzymes (Giedraitienė *et al.*, 2011). Enzymes can change the active site of antibiotic action by cleaving the molecule or adding chemical groups that prevent the antibiotic to act in the target site, resulting in the loss of antibacterial activity (Sandanayakaand Prashad, 2002, Babic *et al.*, 2006). There are three main groups of enzymes that have the capacity to inactivate or modified antibiotics, namely *β*-lactamases, aminoglycoside-modifying enzymes and chloramphenicol acetyltransferases (Giedraitienė *et al.*, 2011).

The hydrolysis of antibiotics mediated by *β*-lactamases correspond to one of the most important mechanism of resistance to *β*-lactam, which has serious implications for infection treatment, particularly in hospital and veterinary medicine (Woodford *et al.*, 2011, Arenas-Marquez *et al.*, 2012, Johnsonand Woodford, 2013, Voulgari *et al.*, 2013). *β*-lactamases cleave the *β*-lactam ring of *β*-lactam antibiotics (Bush *et al.*, 1995), being classified by two systems: (i) the Amber classification and (ii) the Bush-Jacoby-Medeiros (Alekshunand Levy, 2007, Bushand Jacoby, 2010).

More than 300 different *β*-lactamases are described (Wickensand Wade, 2005), and according to their amino acid sequence (Ambler classification) there are four different classes of *β*-lactamases, of which classes A, C and D have active-site serine enzymes (Ambler, 1980). The class B includes metallo *β*-lactamases (MBLs), which have zinc ion in their active site

(Walsh, 2000). The functional classification of *β*-lactamases is based on their catalytic properties, which gives the chance to relate them to their clinical impact.

The aminoglycoside modifying enzyme (AMEs) represents another class that inactives the antibiotic. Unlike *β*-lactamases, aminoglycoside resistance is mediated by modifications in their chemical structure by binding adenylyl, phosphoryl or acetyl groups to the periphery of the antibiotic molecule (Stratevaand Yordanov, 2009). There are three families of aminoglycoside-modifying enzymes: phosphoryltransferases (APHs), nucleotidyltransferases (ANTs) or adenylyltransferases (AACs) (Walsh, 2000).

2.4.3 Antibiotic target modification

A crucial feature of the target site for the antibiotic action is their vital role in bacteria survival and growth, and an interference with bacteria function is either inhibitory of bacterial growth or lethal to the cell. In selective antibiotics for human therapy the target of antibiotic action must also be absent from human cell or if present must differ from the target of antibiotic action (Lambert, 2005). The interaction between a target molecule and an antibiotic occurs with high affinity. So, even one small modification in a target molecule can influence the antibiotic binding. Consequently, modification to the target structure that inhibit the efficient antibiotic binding, but that still enable the target to carry out its normal cellular functions, can confer resistance (Giedraitienė *et al.*, 2011).

Bacterial cell wall, constituted by peptidoglycan is an excellent example of a selective target. Peptidoglycan has a composition and chemical structure unlike any mammalian macromolecule, and it is important to the growth and survival of the most bacteria (Giedraitienė *et al.*, 2011). Glycopeptides (e.g. vancomycin) and *β*-lactams (e.g. penicillin) are antibiotics capable of inhibiting the cell wall synthesis, however, *β*-lactams have affinity to transpeptidases, while glycopeptides have affinity to transglycosylases. The presence of a modification in these enzymes reduces the antibiotic affinity, resulting in microbial resistance (Lambert, 2005). In Gram-positive bacteria, mutations and/or reduction of expression alterations in penicillin-binding proteins (PBPs) are the most important mechanisms of resistance (Beceiro *et al.*, 2013).

In antibiotic resistant clinical pathogenic bacteria are found several types of target alteration which confer resistance to a few antibiotic, such as to sulphonamides and to trimethoprim (Spratt, 1994). In this mechanism bacteria acquire genes that encode new target enzymes that have lower affinity for the antibiotic than the normal enzyme (Spratt, 1994).

In the last years, alteration of the antibiotic target site has been found to be a clinical important mechanism of resistance to relevant clinical antibiotics, namely the erythromycin ribosome methylase (*erm*) family of genes methylate 16S rRNA that alter the antibiotic binding site, preventing the binding of streptogramins, macrolides and lincosamines. The modification the binding of antibiotics by methylase reduces, which results in resistance against streptogramins, macrolides and lincosamines (Kumar *et al.*, 2014).

2.5 BIOFILMS

2.5.1 Occurrence and architecture of bacterial biofilms

The perception of bacteria as a single cell has been exploited and used to study many of bacterial activities for a long time, although this traditional way of unicellular life of bacteria is rarely how bacteria exist in nature. In the major natural environments, bacterial cells live predominantly in dense structures, named biofilms (Watnickand Kolter, 2000). These structures appear early in the fossil record, particularly in hydrothermal environments, and are common not only in bacteria but also on Archaea and Eukaryotic microorganisms such as fungi and protozoa (Hall-Stoodley *et al.*, 2004).

Environmental microbiologists soon accepted that organized bacterial communities are responsible for driving the biogeochemical cycling that maintains the biosphere (Makinand Beveridge, 1996). *In situ* observations of several natural habitats has established that the majority of the microorganisms persists by adhering to a substratum within a structured biofilm ecosystem and not as free-single cell (Costerton, 1995).

Biofilms are defined as a complex community of microbial cells attached to a biotic or abiotic surface, or associated with interfaces. Biofilms can exits on all types of surface such as tissues, medical devices (implants and prosthetic, catheters and dental material), and industrial surfaces (Donlan, 2002). According to DonlanandCosterton (2002) a biofilm is a complex, cooperating and organized microbial community constituted by cells that are irreversibly adhered to a surface or interface or each other, are embedded in a hydrated matrix of extracellular polymeric substance (EPS) that themselves have produced, and exhibit an altered phenotype through respect to gene transcriptions and growth rate. Some of this sophisticated structure is sufficiently dense to be visible to the naked eye (O'Toole *et al.*, 2000).

The recent definitions of Biofilms definitions take into account not only the readily observable characteristics, such as cell irreversibly attached to an surface, surrounded by an

extracellular matrix with polymeric substances, and including the non-cellular or abiotic constituents, but also several physiological characteristic of the microorganisms, such as the altered growth rate and the fact that biofilm microorganisms transcribe genes that planktonic microorganisms do not (Donlanand Costerton, 2002).

Biofilms can be composed by a population that is developed from a single-species or a population derived from multiple microbial species (Donlan, 2002). Multiple microbial species biofilms are predominate in the environment, while single-species biofilm are most commonly described in surface of medical implants and infectious diseases (Adaland Farr, 1996).

Biofilms are ubiquitous and can develop in almost every environment and man-made surface (Parsekand Singh, 2003). In the environment, biofilms can develop under extreme conditions (Hall-Stoodley *et al.*, 2004), for example, some works reported the presence of biofilms inhabiting thermal water (35-50 ºC), while other research groups have found such structures on frozen glaciers (Yang *et al.*, 2009). These communities can also be found living in extreme acidic environments, characterized by a high metal content and lack of nutrients (Dufour *et al.*, 2012).

The formation of biofilms occurs in response to environmental changes and comprises a complex and multiple regulatory systems (Garrett *et al.*, 2008). This is produced in order to protect the microorganisms from environmental stresses such as that encountered in host tissue (antibodies, phagocytes, etc.) or on an inert surface exposed to inhospitable conditions (desiccation, oxygen toxicity, starvation, UV light, heat, cold, shear forces) and antibiotic agents (Costerton *et al.*, 1995, O'Toole *et al.*, 2000, Mahand O'Toole, 2001). The formation of biofilms by single or multi-species, is a process that requires collective bacterial behaviour (Daveyand O'toole, 2000), and all behaviour patterns are organized by communication, which can be chemical or genetic (Weigel *et al.*, 2007).

The transition from planktonic lifestyle to sessile communities occurs as a consequence of environmental changes that trigger the dysregulation of complex regulatory networks. Thus, upon sensing stress signal, planktonic microorganisms will initiate attachment to a surface, which will lead to the formation of a biofilm that has a greater ability to resist to environmental challenges (Costerton *et al.*, 1999, O'Toole *et al.*, 2000, Hall-Stoodley *et al.*, 2004). The growth of the biofilm community is slow, and cells in different sections of the biofilm structure show different patterns of genes expression (Whiteley *et al.*, 2001).

Investigators indicate that the biofilm structures is a stable point in a biological cycle that includes four steps: (i) initiation, (ii) maturation, (iii) maintenance and (iv) dissolution (O'Toole *et al.*, 2000). In the natural environment, bacteria do not attach directly to a surface *per se*, but in effect attach to a conditioning film, which is known to form on most surfaces. The conditioning film has a complex composition that result from a chemical modification of the original substratum, thus influencing the rate and extent of bacterial adhesion (Donlan, 2002).

Planktonic cells can adhere to a substratum and initiate biofilm formation in the presence of shear forces. This initially reversible attachment can occur through adhesion structures, including flagella, pili and external microbial layers, or due to immobilization of the bacterial cells, that requires physical and electrostatic interactions between the cell and the surface (Landry *et al.*, 2006). The adhesion of microorganisms to a substratum depends on a numbers of bacterial cells, and physical, chemical and surface material-parameters. Especially, the material topography has been widely discussed as an important characteristic influencing bacterial cells adhesion (Flint *et al.*, 1997).

If the microbial cells are not immediately separated from the surface, the cells can anchor themselves more firmly/permanently and grow. The adhesion to a solid surface induces the expression of a bacterial enzymes, which catalyzes the development of exopolysaccharides that promote colonization and protection (Pavithraand Doble, 2008).

Once a biofilm is formed, and the exopolysaccharide matrix has been produced by the attached cells, the resultant complex structure is highly viscoelastic and behaves as rubbery manner. The EPS is constituted by polysaccharides, proteins and DNA originated from bacterial cells (Wingender *et al.*, 2001, Whitchurch *et al.*, 2002, Costerton *et al.*, 2003). Non-cellular constituents, such as mineral crystal, corrosion particles, clay or silt particles, or blood components, depending on the environment under which the bacterial biofilm has developed, may also be found in the biofilm extracellular matrix (Donlan, 2002). The biofilms EPS is important since it provides structural stability and protection to the biofilm community (Hoiby *et al.*, 2010).

EPS plays important roles in function and structure of different biofilms communities. This extracellular matrix provides certain degree of shelter on homeostasis to the cells residing in biofilms. EPS has been reported to provide protection from several environmental stress such as pH shift, UV radiation, osmotic shock and desidratation (Daveyand O'toole, 2000).

The structure that form biofilms contain channels in which nutrients help to distribute nutrients and signalling molecules (Werner *et al.*, 2004). These water channels offers active

exchanging nutrients and metabolites with the bulk aqueous phase, enhancing the nutrient availability as well as removal of potentially toxic metabolites. This multicellular structure creates chemical and metabolite gradients and heterogeneity in oxygen availability (Watnickand Kolter, 2000). Studies of *in vivo* and *in vitro* biofilms have shown that the oxygen concentration can be high on the surface, but low in the centre of the biofilms where anaerobic conditions may present (Hoiby *et al.*, 2010).

Once the mature biofilm has formed, some cells within the population start to dissociated from the biofilm structure. This step is denominated as dispersal, and is essential to complete the biofilms cycle by enabling the cell to spread and colonized new surface (O'Toole *et al.*, 2000).

In microorganisms, the production of several extracellular factors, including those involved in biofilm development are controlled by quorum sensing (QS) systems. QS is a bacterial communication system, which allows individuals to monitor the overall population density of the group. QS involves the production, release and reception of extracellular signalling molecules, called autoinducers. At sufficient cell densities, the signalling molecules accumulate to concentrations needed for expression of genes coding for extracellular factors (Williams *et al.*, 2007, von Bodman *et al.*, 2008). The autoinducers are different between Gram-negative and -positive bacteria. Peptide with small size are the QS molecules described in Gram-positive bacteria, while *N*-acyl homoserine lactone (AHL) are the predominant QS molecules of Gram-negative bacteria (Jensen *et al.*, 2007).

2.5.2 Antibiotic resistance in biofilms

Biofilm have been widely studied over the last years in part because they are responsible for 65% or more of all infectious diseases, principally in device-related infections and chronic infectious diseases (Costerton *et al.*, 1999, O'Toole *et al.*, 2000, Hall-Stoodley *et al.*, 2004). They are particularly important because of their resistance to host defence mechanisms and to conventional antibiotic therapy (Kolter, 2010).

Biofilm structural nature and the characteristics of the sessile bacterial cells, produce resistance towards the antibiotics, leading to a protected environment against adverse condition and hosts defences mechanisms (Costerton *et al.*, 2003, Patel, 2005). Biofilm possesses an increased survival and resistance to environmental and chemical stressors (Hoiby *et al.*, 2010).

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of antibiotic to biofilms is up to 100 - 1000 fold higher than free bacterial cells, and may be 150 - 3000 times more resistant to disinfectant compounds (Mahand O'Toole, 2001, Patel, 2005).

Biofilm reduced susceptibility is result of a combination of different factors (Figure 2.6), including physical or chemical diffusion barriers to antibiotic penetration into the biofilm structure, slow growth of the biofilm owing to nutrient limitation, activation of the general stress response and the emergence of a biofilm specific phenotype (Mahand O'Toole, 2001). It is possible to separate antibiotic resistance mechanisms of biofilm into innate (or intrinsic) and induced (or extrinsic) (Andersonand O'toole, 2008).

The innate factors of antibiotic resistance are activated as part of the biofilm formation cycle, which are integral parts of the biofilm physiology and structure resulting from conversion to a biofilm community. The impact of several different innate biofilm factors affecting antibiotic resistance have been recognized (Mahand O'Toole, 2001, Hoiby *et al.*, 2010).

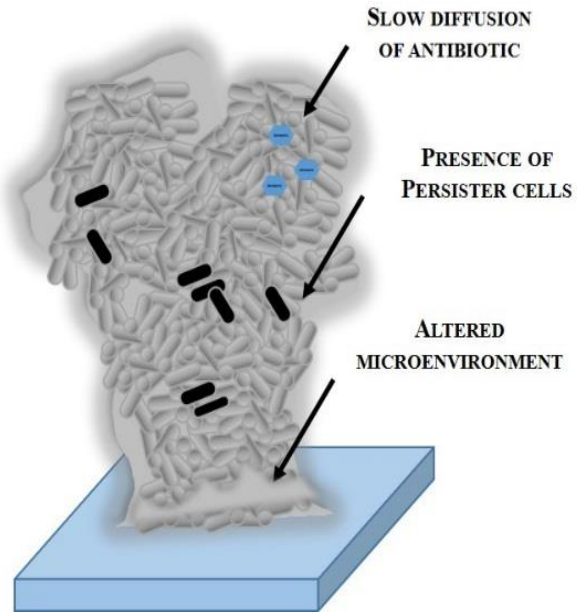

Figure 2.6 Biofilms mechanisms of antibiotic resistance. Adapted from Borges *et al.* (2016).

Other groups of antibiotic resistance factors are the ones resulting from transcriptional induction by treatment with the antibiotic itself, inducing resistant factors (Andersonand O'toole, 2008).

The mutation frequency biofilm development is significantly increased compared with free-floating bacteria and there is an increased horizontal gene transmission in biofilm. These physiological characteristics may expound why biofilm developing bacteria easily became multiresistant by means of common resistance mechanisms against *β*-lactam antibiotics, fluoroquinolones and aminoglycosides, which may be detected by a routine susceptibility assay in a clinical laboratory. Thus, biofilms can simultaneously produce enzymes that degrade antibiotic, have antibiotic targets of low affinity and over express efflux pumps that have a broad range spectrum (Costerton *et al.*, 2003, Macfarlaneand Macfarlane, 2007).

Delayed penetration of the antibiotic on biofilm

Antibiotic compounds must diffuse through the biofilm EPS in order to inactivate the enclosed bacterial cells. This exopolymeric matrix in addition to providing structural stability, possesses a diffusional barrier that prevent the access of different agents, including antibiotic molecules, to the bacterial cells (Mahand O'Toole, 2001).

The study of Suci *et al.* (1994) on the subject of antibiotic diffusion, using infrared spectroscopy, demonstrated a delayed penetration of the antibiotic ciprofloxacin through *P. aeruginosa* biofilm, which typically required 40 s for a sterile surface, required 21 min for biofilm-containing surface.

Initially, it was thought that the delayed penetration was due to a filtration effect such than the antibiotic molecules failed to effectively penetrate the biofilm. However, some studies showed that the EPS has large water-filled spaces as well as channels (Kumon *et al.*, 1994, Singh *et al.*, 2010). On the other hand, the negatively charged EPS is also very effective in preventing the access of positively charged molecules such as aminoglycoside antibiotics to the bacterial cells, by restricting their permeation, possibly through binding. However, not all antibiotic molecules are equally affected (Shigeta *et al.*, 1997, Ishida *et al.*, 1998). Dunne *et al.* (1993) demonstrated that *S. epidermidis* biofilm formed a membrane allowing the diffusion of vancomycin and rifampicin across the biofilm, implying that these molecules could efficiently penetrate these structures. These studies suggest that diffusion limitation cannot always explain biofilms resistance to antibiotics. However, it is possible that EPS are able to increase the concentration of antibiotic-degrading enzymes (Mahand O'Toole, 2001).

The synergy between delayed penetration and degradation provides effective resistance. One the other hand, the synergistic arrangement between the delayed penetration and an enzyme accumulation is analogous to the effective synergy between the outer

membrane and multiresistant pumps that transport antibiotic molecules across this permeability barrier (Lomovskayaand Lewis, 1992, Nikaido, 2003). Increasing evidences indicated that multiple mechanisms are required for the overall biofilm antibiotic resistance (Mahand O'Toole, 2001).

Altered growth rate of bacterial biofilm

Biofilm are very complex and typically consist of a heterogeneous communities of cell in different growth rate. Even in single-species biofilms, the population is heterogeneous, which cover the spectrum from rapidly growing to metabolically inactive (de la Fuente-Nunez *et al.*, 2013).

Most antibiotics are active in actively growing bacteria, an example is penicillin which is only active on growing cells. Most antibiotics target some types of macromolecular synthesis, and for this reason antibiotics would not be expected to have much effect on bacteria in which macromolecular synthesis is stopped (Stewart, 2002).

When a bacterial cell experience some nutrient limitation it slows its growth. Alteration from exponential to slow or no growth is commonly accompanied by an increase in resistance to antibiotics (Mahand O'Toole, 2001).

It was demonstrated that microgradients of key metabolic substrates and products occur inside biofilms. For this reason biofilms include slow-growing or stationary-phase cells (Stewart, 2002). Slow growth was observed in mature biofilms (Wentland *et al.*, 1996). Bacteria metabolically inactive are uniquely well positioned to resist antibiotic challenge, and are more resistant than a biofilm in which all of the cells grow at a uniform intermediate rate (Xu *et al.*, 2000, Stewart, 2002).

Pamp *et al.* (2008) found that fluoroquinolones and tetracycline were only active in the metabolically active cells in the upper layers of a *P. aeruginosa* biofilm. Another study showed that the slowest growing *E. co*li biofilm was the most resistant to cetrimide (Evans *et al.*, 1990). Duguid and collaborated (1992) found that *S. epidermidis* biofilm growth rates strongly influenced susceptibility, the faster the rate of bacteria growth, the more rapid the rate of inactivation by ciprofloxacin.

Presence of persister cell

Chemical, physiological, and genetic diversity of biofilms increases over both space and time (Stewartand Franklin, 2008). Biofilm cells can exist in at least four distinct states: aerobic growth, fermentative growth, dead and dormant (Rani *et al.*, 2007).

Dormant or persisters cells are in a particular physiological state with non-growing or low-growing and with low levels of translation, but an unique gene expression profile, associating the switch off of gene encoding metabolic proteins together with operons encoding toxin-antitoxin pairs switched on (Shah *et al.*, 2006, de la Fuente-Nunez *et al.*, 2013).

Persisters cells, can withstand the presence of otherwise inhibitory concentrations of antibiotic, and are more able to survive stressful conditions, including antibiotic challenges (Lewis, 2008). These cells may constitute a relatively small fraction of the biofilm population, but presister cells have entered a highly protected, maybe spore-like, state. These cells are not mutants, but rather phenotypic variants of the wild-type (Lewis, 2001, Stewartand Costerton, 2001).

Expression of possible biofilm-specific resistance genes

Horizontal gene transfer is important for the evolution and genetic multiplicity of natural microorganisms (Aminov, 2011). Acquisition of new genetic trait provide chances to the microbial community transcribe the necessary genes to becomeactive member of the biofilms structure (Aminov, 2011). During biofilm formation, specific traits can be expressed that can confer antibiotic resistance, which is not found in planktonic bacterial cells (Mahand O'Toole, 2001). For example, β-galactosidase was observed to be expressed in reaction to imipenem and piperacillin in biofilms of *P. aeruginosa* (O'Toole *et al.*, 2000).

CHAPTER 3. MATERIAL AND METHODS

3.1 SAMPLE COLLECTION, BACTERIAL ISOLATION AND GROWTH CONDITIONS

A total of 140 faecal samples from 36 different wild animal species, including birds, reptiles and mammals (Table 3.1), were collected aseptically, directly from animal rectum or cloaca, or from freshly voided faecal material. The collection was done as soon as the animal entered in the *Centre for Reception, Recovery and Treatment of Wild Animals* (CRATAS) of the University of Trás-os-Montes and Alto Douro and had taken care to avoid cultured contamination with environmental material. Faecal samples were enriched in 5 mL of Brain Heart Infusion (BHI) broth (Oxoid, UK) for 24 h at 30 ºC.

Table 3.1 Diversity of faecal samples used in this study

Animal	**Animal common name**	**Nº of samples collected**
Bubo bubo	Eagle owl	2
Buteo buteo	Common buzzard	18
Vulpes vulpes	Red fox	7
Strix aluco	Tawny owl	17
Tyto alba	Barn owl	4
Amazona aestiva	Blue-fronted Amazon	2
Oryctolagus cuniculus	Black rabbit	7
Accipiter nisus	Sparrow hawk	2
Milvus migrans	Black kite	1
Circaetus gallicus	Short-toed snake eagle	1
Ciconia ciconia	White stork	4
Psittacidae	Parrots	1
Luscinia megarhynchos	Nightingale	1
Capreolus capreolus	Roe deer	2
Rhea americana	Greater rhea	1
Passeriformes	'Passerine' birds	1
Perdix perdix	Grey partridge	2
Apodemus sylvaticus	Wood mouse	1
Sciurus vulgaris	Red squirrel	5
Apus apus	Common swift	1
Athene noctua	Little owl	2
Mauremys leprosa	Spanish turtle	1
Colubridae	Snake	3
Streptopelia turtur	Turtle dove	1
Coturnix coturnix	Common quail	6
Gyps fulvus	Griffon vulture	1
Accipiter gentilis	Northern goshawk	4
Genetta genetta	Common genet	2
Sus scrofa	Wild boar	4
Pipistrellus pipistrellus	Common pipistrelle	9
Melopsittacus undulatus	Budgerigar	1
Pavo cristatus	Indian peafowl	1
Ardea cinerea	Heron	2
Falco peregrinus	Peregrine falcon	1
Columba livia	Rock pigeon	12
Cervus elaphus	Red deer	10

Direct streaking of faecal swabs on selective and differential culture media was used to select, strains and purify strains by incubating plates aerobically at 30 ºC for 24 h. All strains were checked for oxidase (1% tetramethyl phenylenediamine, Merck, Germany) activity before cryopreservation in BHI with 15% glycerol at -70 ºC. Working cultures were routinely grown aerobically at 30 °C in BHI.

3.2 STRAIN IDENTIFICATION

3.2.1 API 20 NE system test

The biochemical profile of bacterial strains was determined using the standard classification technique API 20NE (BioMérieux, France), following the manufacturer's instructions. This test allows non-fastidious, non-enteric Gram-negative rods, to be classified according to their ability to utilize 20 different substrates.

3.2.2 Identification by sequencing

3.2.2.1 DNA extraction

Total genomic DNA was extracted from overnight pure colonies as previously described by Soler *et al.* (2004). A distinct colony was resuspended in 100 µL of Tris-EDTA (TE) buffer and 200 µL of chelex at 20% (Bio-Rad, USA) and was vortexed for 1 min. Cycles of 10 min at 95 ºC, followed by 10 min at -20 ºC, were repeated three times. The mixtures were centrifuged at 15 856 g in a 5415D Eppendorf (Eppendorf, Germany) centrifuge, for 5 min. The supernatant was transferred to another tube and stored at -20 ºC until use.

3.2.2.2 PCR reaction

Polymerase chain reaction (PCR) mixtures (50 µL) contained 2 mM of $MgCl_2$, 50 mM of KCl, 75 mM of Tris HCl (pH 9.0), 20 mM of $(NH_4)_2SO_4$, 2.5 mM of each dNTP, 10 pmol of each primer, 1 µL (1U) of *taq* DNA polymerase (Biotools, Spain) and 1 µL of template genomic DNA.

The mixtures were submitted to different amplification conditions according to the gene to be amplified. The conditions for gene amplification were for the 16S rRNA: initial denaturation at 94 ºC for 5 min, followed by 35 amplification cycles composed of a

denaturation step at 94 ºC for 15 seconds, an annealing step at 55 ºC for 30 seconds and an extension period at 72 ºC for 1 min and 30 seconds. Finally, the mixture underwent a cycle of final extension at 72 ºC for 5 minutes.

For amplification of the *gyr*B gene the following conditions were: initial denaturation for 5 min at 94 ºC, followed by 35 amplification cycles composed of a denaturation step at 94 ºC for 15 seconds, an annealing step at 55 ºC for 30 seconds and an extension period at 72 ºC for 45 seconds. Finally, the mixture underwent a cycle of final extension at 72 ºC for 5 minutes.

The oligonucleotides used to amplify the *gyr*B and 16S rRNA genes are described in Table 3.2.

Table 3.2 Primers used for PCR amplification and sequencing of *gyr*B and 16S rRNA genes

Primer pair	Position	Sequence 5`-3	Reference
*gyr*B 3F	334/354*	TCC GGC GGT CTG CAC GGC GT	Yáñez *et al.* (2003)
*gyr*B14R	1464/1444*	TTG TCC GGG TTG TAC TCG TC	Yáñez *et al.* (2003)
16S 0F	8/27[π]	AGA GTT TGA TCA TGG CTC AG	Martínez-Murcia *et al.* (1999)
16S 15R	1492/1510[π]	GGT TAC CTT GTT ACG ACT T	Martínez-Murcia *et al.* (1999)

*Position according to *E. coli* numbering (Huang, 1996); [π] Position according to *E. coli* numbering (Stern el al., 1988).

The amplification products were subjected to agarose gel electrophoresis (Sigma-Aldrich, Portugal) prepared in 1% Tris-acetate-EDTA buffer (TAE) and adding ethidium bromide (Invitrogen, UK) to 5 μg/ mL. 4 μL of each PCR reaction mixed with 2 μL loading buffer (Invitrogen, UK) and a voltage of 6 V/ cm was applied for 20 minutes. Amplified products were purified with QIAquick PCR purification kit (QIAGEN, Germany), following the manufacturer's instructions.

3.2.2.3 Sequencing reaction

The purified amplification products were prepared with the Big Dye® Terminator Cycle Sequencing kit (Applied Biosystems, UK), according to the manufacturer's recommendations. The sequencing reaction was prepared to a final volume of 10 μL, adding 4 μL of the Premix solution, 4 μL of genomic DNA and 2 μL of the primer at 2 μM. The oligonucleotides used are shown in Table 3.3.

Table 3.3 Oligonucleotides used in the sequencing reaction

Primer pair	Position	Sequence 5`-3	Reference
*gyr*B 3F	334/354*	TCC GGC GGT CTG CAC GGC GT	Yáñez *et al.* (2003)
*gyr*B 7R	792/812*	GGG GTC TAC TGC TTC ACC AA	Yáñez *et al.* (2003)
16S 0F	8/27$^{\pi}$	AGA GTT TGA TCA TGG CTC AG	Martínez-Murcia *et al.* (1999)
16S 9R	926/908$^{\pi}$	CCG TCA ATT CAT TTG AGT TT	Martínez-Murcia *et al.* (1999)
16S 15R	1492/1510 $^{\pi}$	GGT TAC CTT GTT ACG ACT T	Martínez-Murcia *et al.* (1999)

*Position according to *E. coli* numbering (Huang, 1996); $^{\pi}$ Position according to *E. coli* numbering (Stern *et al.*, 1988).

The products of the sequencing reaction were precipitated according to the protocol listed in Table 3.4.

Table 3.4 Protocol for precipitation sequencing products

1. In a tube (1.5 mL) place: 2.5 µL EDTA (125 mM) and 30 µL 100% ethanol
2. Add 10 µL of the sequencing reaction, mix by inversion and leave at room temperature for a period of 15 minutes
3. Centrifuge 20 minutes at 17 075 g at 4 ºC
4. Eliminate the solution of ethanol and EDTA
5. Add 100 µL of 70% ethanol
6. Centrifuge for 2 minutes at 17 075 g at 4 ºC and eliminate the entire ethanol solution
7. Dry the pellet (10 minutes in the Concentrator - Vacufuge, Eppendorf, Germany)
8. Store the pellet dry at -20 ºC
9. Resuspend the pellet in 20 µL of deionized formamide, and leave for 5-10 minutes at room temperature
10. Vortex for 2 minutes and centrifuge at 15 856 g for 1 minute
11. Analyse in the ABI3100 Avant Genetic Analyzer (Applied Biosystems, UK) automated sequencer plate

The sequencing service was carried out at the Molecular Diagnostic Center (MDC) R & D Laboratory, Alicante, Spain (http://www.mdc-bt.com).

3.2.2.4 Sequence analysis and construction of dendrograms

Newly determined sequences were aligned according to previously published reference sequences (Martínez-Murcia *et al.*, 1999, Yáñez *et al.*, 2003, Soler *et al.*, 2004, Saavedra *et al.*, 2006) by the Clustal X alignment program, version 1.8 (Thompson *et al.*, 1997). Genetic distances were obtained by Kimura's 2 parameter model (Kimura, 1980) and the evolutionary tree was constructed by the Neighbour-Joining method (Saitouand Nei, 1987) with the Molecular Evolutionary Genetics Analysis (MEGA) program, version 2.1 (Kumar *et al.*, 2001).

3.3 ANTIMICROBIAL DRUG SUSCEPTIBILITY TESTING

3.3.1 Antimicrobial susceptibility disk diffusion method

The antimicrobial drug susceptibility of strains was determined by the disk method of Kirby-Bauer (Bauer *et al.*, 1966) on Mueller-Hinton agar plates (MHA, Merck, Germany) according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014), with inocula adjusted to an optical density (OD) of 0.5 McFarland standard units.

After 24 h of incubation at 30 ºC, organisms were classified as sensitive (S), intermediate (I), or resistant (R). The following antibiotic-containing discs were obtained from Oxoid:

- ***ß*-lactams**: amoxicillin (AML_{10}), amoxicillin/clavulanic acid (AMC_{30}), ticarcillin (TIC_{75}), ticarcillin/clavulanic acid (TIM_{85}), piperacillin (PRL_{100}), piperacillin/tazobactam (TZP_{110}), cephalothin (KF_{30}), cefoxitin (FOX_{30}), ceftriaxone (CRO_{30}), cefoperazone (CFP_{30}), ceftazidime (CAZ_{30}), cefotaxime (CTX_{30}), cefepime (FEP_{30}), imipenem (IPM_{10}), aztreonam (ATM_{30});
- **Aminoglycosides**: streptomycin (S_{10}), kanamycin (K_{30}), amikacin (AK_{30}), gentamicin (CN_{10}),
tobramycin (TOB_{10});
- **Quinolones:** nalidixic acid (NA_{30}), ciprofloxacin (CIP_5);
- **Macrolides**: erythromycin (E_{15});
- **Sulfonamides:** trimethoprim-sulfamethoxazole (SXT_{25});
- **Phenicols**: chloramphenicol (C_{30});
- **Tetracycline (TE_{30});**
- **Fosfomycin (FOS_{50}).**

3.3.2 Determination of minimum inhibitory concentration (MIC)

Standardized planktonic minimum inhibitory concentration (MIC) of CIP (Sigma - Aldrinch Co., Portugal) and IPM (Cayman Chemical., USA) antibiotics was assessed by the microdilution method according to Clinical and Laboratory Standards Institute guidelines (2014) guidelines. Bacterial cells were grown overnight (16-18 h incubation) in batch culture using 100 mL of Muller-Hinton broth (MBH, Merck, Germany) at 30 ± 2 ºC and 150 rpm. Overnight cell cultures were adjusted to cell density of 1×10^6 cells/mL (640 nm) and added to at least 16 wells of a sterile 96-well polystyrene microtiter plates (Orange Scientific, USA)

with the antibiotic in a final volume of 200 µL. The antimicrobial concentrations did not exceed 10% (v/v) of the final volume. Cell suspensions with water and cell suspension without antibiotics were used as controls. The microtiter plates were then incubated for 24 h at 30 ± 2 ºC in an orbital shaker (150 rpm). The absorbance was measured at 640 nm using a Microplate Reader (Spectramax M2e, Molecular Devices, Inc.). MIC corresponded to the concentration in which the final OD was inferior or equal to the initial OD (Testing, 2017).

3.4 GENOTYPIC DETECTION OF *β*-LACTAMASES

3.4.1 Screening of resistance genetic determinants *β*-lactamases

Total genomic DNA was extracted from overnight pure colonies as previously described in section 3.2.2.1. Detection of *β*-lactamase encoding genes was done for TEM-type, SHV-type, CTX-M-type, MOX and FOX variants of extended-spectrum-*β*-lactamases (ESBLs) and for the CphA, IMP-type, VIM-type and OXA-type carbapenemases.

PCR reaction mixtures (50 L) contained 5 µL of Biotools PCR buffer (10×), 200 µM of each nucleotide, 10 pmol of each primer (Table 3.5), 1 µL (1U) of *taq* DNA polymerase (Biotools, Spain) and 1 µL of template genomic DNA.

Table 3.5 Primers used for PCR amplification and sequencing

Target	Primer pair	Sequence 5`-3	Amplicon size (bp)	Reference
bla$_{TEM}$	TEM-F	CTG GAT CTC AAC AGC GGT AAG	403	Fontes (2009)
	TEM-R	ACG TTG TTG CCA TTG CTG CAG		
bla$_{SHV}$	SHV-F	TTC GCC TGT GTA TTA TCT CC	373	Fontes (2009)
	SHV-R	GTT ATC GCT CAT GGT AAT GG		
bla$_{CTX-M}$	CTX-BF	GCC TGC CGA TCT GGT TAA C	209	Fontes (2009)
	CTX-BR	GGA ATG GCG GTA TTC AGC G		
bla$_{MOX}$	MOX-F	CGT GCT CAA GGA TGG CAA G	634	Fontes (2009)
	MOX-R	CTG CTG CAA CGC CTT GTC A		
bla$_{FOX}$	FOX-F	TGT TCG AGA TTG GCT CGG TC	282	Fontes (2009)
	FOX-R	GGG TTG GAA TAC TGG CGA TG		
bla$_{CphA}$	CphA-3F	CTG GAG GTG ATC AAC ACC A	410	Fontes (2009)
	CphA-7R	TTG ATC GGC AGC TTC ATC GC		
bla$_{VIM}$	VIM-F	GGT GTT TGG TCG CAT ATC GC	195	Fontes (2009)
	VIM-R	CAT GAA AGT GCG TGG AGA CTG		
bla$_{OXA}$	OXA-AerF	GAC TAC GGC AAC CGG GAT C	215	Fontes (2009)
	OXA-AerR	CTT GCC GTG GAT CTG CCA G		
	OXA-BF	GAT AGT TGT GGC AGA CGA ACG	453	
	OXA-BR	CTT GAC CAA GCG CTG ATG TTC		
	OXA-CF	GTT CTC TGC CGA AGC CGT CA	554	
	OXA-CR	GAC TCA GTT CCC ACA CCA G		
bla$_{IMP}$	IMP-F	GAAGGCGTTTATGTTCATAC	559	Fontes (2009)
	IMP-R	CTTCACTGTGACTTGGAAC		

The conditions for gene amplification were: initial denaturation at 95 ºC for 5 min, followed by 35 amplification cycles composed of a denaturation step at 94 ºC for 15 seconds, an annealing step at 55 ºC for 30 seconds (except for bla_{FOX}, in which 60 ºC were used) and an extension period at 72 ºC for 45 seconds. Finally, the mixture underwent a cycle of final extension at 72 ºC for 3 minutes.

3.5 PRODUCTION OF SIDEROPHORES, PROTEASES AND GELATINASES

The strains were obtained from overnight cultures grown in 100 mL tubes with 50 mL of Trypticase Soy broth (TSB, Merck, Gernsheim, Germany), incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Göttingen, Germany). An $OD_{600\ nm}$ of 0.2 was obtained in a test tube with saline solution at 8.5 g/ L. At least three independent experiments were performed for all the tests.

3.5.1 Siderophores

Chrome azurol S (CAS) agar plates were prepared according to the method used by SchwynandNeilands (1987). Microbial suspension (10 µL) were placed in each 90 mm plate of CAS agar plates (in three different positions). The plates were incubated at 30 ºC, for 48 h. After the incubation time an orange halo was measured, indicating the production of siderophores (Schwynand Neilands, 1987, Neilands, 1995, Milagres *et al.*, 1999).

3.5.2 Proteases

Microbial suspensions (10 µL) were placed in 90 mm plate (in three different positions) of Plate Count Agar (PCA, Oxoid, England) with 1 g/ L skim milk powder. The plates were incubated at 30 ºC, for 72 h. After the incubation time, the plates were flooded with 1.00 mol/ L hydrochloric acid. The clearance zones formed indicated protease positive strains (Doganand Boor, 2003).

3.5.3 Gelatinases

Microbial suspensions (10 µL) were placed in 90 mm plate (in three different positions) of gelatin agar plate (5 g/ L peptone, 3 g/ L yeast extract, 30 g/ L gelatin, 15 g/ L

agar, at pH 7). The plates were incubated at 30 ºC, for 48 h. After the incubation period, the plates were flooded with 2.84 mol/ L ammonium sulphate. If the bacteria produced gelatinase, a transparent halo around cells appears (gelatin precipitates) (Lopes Mde *et al.*, 2006).

3.6 BIOASSAY FOR DETECTION OF *N*-ACYL HOMOSERINE LACTONES (AHL) PRODUCTION

To detect de production of *N*-acyl homoserine lactones (AHLs) (C4-HSL - *N*-butanoyl homoserine lactone) and C6 - *N*-hexanoyl homoserine lactone), molecules integral to bacterial quorum sensing (QS) an assay was performed according to McLean *et al.* (2004). The strains demonstrating capacity to inhibit QS were streaked on Luria-Bertani (LB, Liofilchem, Italy) agar plates and incubated overnight at 30 ºC. The indicator strain *Chromobacterium violaceum* CVO26 was grown overnight in LB broth. *C. violaceum* CVO26 is sensitive to C4 - HSL and C6 - HSL. After overnight grown the strains were overlaid in an optical density (OD) of 0.1 at 600 nm and the plates were incubated for 24 h at 30 ºC. A positive resulted was indicated by a purple pigmentation of *C. violaceum* CVO26 around the strains (McLean *et al.*, 2004). Three independent assays were performed.

3.7 SURFACE HYDROPHOBICITY AND FREE ENERGY OF ADHESION

Hydrophobicity was evaluated after contact angles measurement, as described previously by van Oss *et al.* (1987, 1988, 1989). For the determination of contact angles, strains were prepared according to the method described by Busscher *et al*. (1984). Bacterial suspensions were centrifuged for 10 min at 3772 g and washed three time with ultrapure water (pH 6). The strains was adjusted to 0.4 ± 0.02 ($OD_{640\ nm}$). Their physicochemical properties were determined by the sessile drop contact angle measurement on bacterial lawns. For preparation the strains, fifty milliliters of each strains suspension was filtered using a sterile cellulose nitrate membrane filter (pore size 0.22 μm) (Whatman, England).

In this approach, the degree of hydrophobicity of a given surface (*s*) is expressed as the free energy of interaction between two entities of that surface, when immersed in water (*w*) – (ΔG_{sws} in mJ/ m^2). When the interaction between the two entities is stronger than the interaction of each entity with water the $\Delta G_{sws} < 0$ mJ/ m^2 and the material is considered hydrophobic. Conversely, if $\Delta G_{sws} > 0$ mJ/ m^2, the material is hydrophilic. ΔG_{sws} can be calculated through the surface tension components of the interacting entities, according to

Equation 1:

$$\Delta G_{sws} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_W^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+} - \sqrt{\gamma_s^+\gamma_s^-} - \sqrt{\gamma_w^-\gamma_w^+}\right) \quad (1)$$

where γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}), with $\gamma^{AB} = 2 \times \sqrt{\gamma^+\gamma^-}$. The surface tension components of a surface (*s*) are obtained from the contact angles of the three liquids (*l*): water, formamide and α-bromonaphthalene (Sigma, Portugal), were used to define the surface tension of the bacterial surface (Table 3.6). Once the values are obtained, three equations (one for each liquid) of the type below can be solved:

$$(1 + \cos\theta)\gamma_w^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_w^{LW}} + \sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+}\right) \quad (2)$$

where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$. The free energy of adhesion was determined through the surface tension components of the entities involved in the adhesion process by the thermodynamic theory expressed by Dupré equation (3). When studying the interaction between one bacterium (*b*) and a substratum (*s*) that are immersed or dissolved in water (*w*), the total interaction energy - ΔG_{bws}^{TOT}, can be expressed by the interfacial tension components as:

$$\Delta G_{bws}^{TOT} = \gamma_{bs} - \gamma_{bw} - \gamma_{sw} \quad (3)$$

For instance, the interfacial tension for one diphasic system of interaction (bacterium/substratum - γ_{bs}) can be defined by the thermodynamic theory according to the following equations:

$$\gamma_{bs} = \gamma_{bs}^{LW} + \gamma_{bs}^{AB} \quad (4)$$

$$\gamma_{bs}^{LW} = \gamma_b^{LW} + \gamma_s^{LW} - 2 \times \sqrt{\gamma_b^{LW} \times \gamma_s^{LW}} \quad (5)$$

$$\gamma_{bs}^{AB} = 2 \times \left(\sqrt{\gamma_b^+ \times \gamma_b^-} + \sqrt{\gamma_s^+ \times \gamma_s^-} - \sqrt{\gamma_b^+ \times \gamma_s^-} + \sqrt{\gamma_b^- \times \gamma_s^+}\right) \quad (6)$$

The other interfacial tension components, γ_{sw} (substratum/water) and γ_{bw} (bacterium/water), where determined in the same way. The value of the free energy of adhesion was obtained by the application of Eqs. 3-6, allowing the assessment of thermodynamic adhesion. Thermodynamically, if $\Delta G_{bws}^{TOT} < 0$ mJ/ m^2 adhesion of one bacterium to a substratum is favorable. If $\Delta G_{bws}^{TOT} > 0$ mJ/ m^2 adhesion is not expected to occur.

Table 3.6 Liquids surface tension components of the liquids used for determination of surface free energy. Adapted from Janczuk et al. (1993)

Surface free energy (mJ/m2)	Liquid		
	Water	**Formamide**	**α-bromonaphalene**
γ^{LW}	21.8	39.0	41.5
γ^{+}	25.5	2.28	1.72
γ^{-}	25.5	39.6	0.0
γ^{TOT}	72.8	58.0	41.5

3.8 BIOASSAY OF COAGGREGATION AND AUTOAGGREGATION

Cultures of study strains were grown under conditions previously shown to produce stationary phase cells by inoculating the strains into 250 mL flasks with 100 mL of MHB overnight (16 -18 h), at 30 ± 2 ºC and under agitation (150 rpm). A visual coaggregation assay, with some modifications from the method of Ledder *et al.* (2008), was used to assess the ability of strains to coaggregate. Strains were harvested by centrifugation at 3772 g for 20 min and resuspended in coaggregation buffer, repeat the washing 3×. This buffer comprised 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.15 M NaCl and 3.1 mM NaN_3 dissolved in 1 mM Tris buffer adjusted to pH 7.0. At the end they were ressuspend to $OD_{600\ nm}$ of 1.5. Equal volumes of each strain suspension were mixed in sterile cuvettes and the optical densities recorded. Autoaggregation was determined using an identical method, by combining two equal volumes of the same strain suspension. The mixtures were left for 1 h at room temperature to allow coaggregation to occur, after which $OD_{600\ nm}$ was once again recorded. The percentage coaggregation was determinate by the following equation:

$$\text{Coaggregation} = \left[\frac{(OD_{600\ nm}\text{before 1h} - OD_{600\ nm}\text{ after 1h})}{OD_{600\ nm}\text{ before 1h}}\right] \times 100 \quad (7)$$

To visualize coaggregation and autoaggregation, the same volume of the suspension ($OD_{600\ nm}$=1.5) (same/different species) was added to a test tube (≈2 mL of each suspension). The cultures were agitated with a vortex for 10 seconds and then the tubes were rolled gently for 30 seconds. The differences at different times were observed under room temperature (Simões *et al.*, 2008).

Classification (Ledder *et al.*, 2008):
+4 - Rapid and complete settling of large coaggregates leaving a water-clear supernatant;
+3 - Coaggregates that settled rapidly but with a supernatant that remained slightly cloudy;
+2 - Coaggregates that formed immediately, but remained suspended in a turbid background;
+1 - Detectable but finely dispersed coaggregates;
0 - Suspensions with no evidence of aggregation or coaggregation.

3.9 INITIAL MONOLAYER ADHESION ABILITY

3.9.1 Substratum

Polystyrene (PS), a hydrophobic substratum, was selected for the adhesion tests. In order to prepare PS for further assay, coupons with dimension of 1 cm × 1 cm were immersed in a solution of 5% (v/v) commercial detergent (Sonasol Pril, Henkel Ibérica S. A.) and in ultrapure water for 30 min with gentle shaking. To remove any remaining detergent, PS was rinsed five times in 2 mL of ultrapure water and subsequently immersed in ethanol at 96% (v/v) for 10 seconds. After being rinsed three time with ultrapure water, PS coupons were dried at 65 ºC for 3 h before being used.

3.9.2 Monolayer adhesion ability

In vitro initial adhesion was determined according to the method of Simões et al. (2007). Sterile microtiter plates (12-well, polystyrene, Orange Scientific, USA) with 2 mL of cell suspension (1×10^6 cells/ mL in MH) and coupons of polystyrene (PS) were incubated at 30 ºC for 2 h in an orbital shaker at 150 rpm. Negative controls were obtained by placing coupons in MHB without bacteria. At the end of the assay, coupons were removed from the wells of the microtiter plates and immersed in 2 mL of sterile saline solution (NaCl, 0.85%) to remove the loosely attached cells.

Afterwards the PS coupons were used for biomass quantification by crystal violet (CV) staining. All the assays were done in triplicate with three repeats.

3.10 BIOFILM ASSAY IN 96-WELL POLYSTYRENE CULTURE MICROPLATES

Biofilm formation in polystyrene 96-well culture microplates was determined using the method developed by Stepanović et al. (2000). Briefly, overnight cultures were adjusted to an initial optical density at 620 nm corresponding to 1×10^8 cells/ mL in MHB and 200 µL aliquots were inoculated into a microplate (a least sixteen wells). The microtiter plates were incubated for 24, 48 and 72 h at 30 ºC and agitated at 150 rpm. The medium (MHB) was carefully and aseptically replaced by fresh one daily. Negative controls wells contained MHB without bacterial cells.

3.11 BIOFILM CONTROL

To determine whether the antibiotic CIP and IPM had activity in biofilm control, microtiter plates with 24 and 48 h old biofilms were exposed to 10× the MIC value, obtained for the two antibiotic, according to Simões et al. (2010). After 1 h and 24 h of exposure, the biofilms were analyzed in terms of biomass and the results are presented as percentages of biofilm reduction and inactivation.

3.12 QUANTIFICATION OF ADHESION/BIOFILMS BIOMASS BY CRYSTAL VIOLET

The biofilm biomass was quantified by CV staining according to Simões et al. (2007). The OD was measured at 570 nm using a Microplate Reader. Biofilm removal percentage (% Br) was given the following equation by:

$$\%BR = \frac{ODc-ODw}{ODc} \times 100 \quad (8)$$

Where the ODc is the value of biofilm non-exposed to the antibiotic and ODw is the value for biofilm exposed to antibiotic. The assays were performed in triplicate with three repeats.

3.13 ADHESION/BIOFILM CLASSIFICATION

Quantification of the bacterial adhesion/biofilm were classified using the scheme of to Stepanović et al. (2000). The classification was based upon the cut-off of the optical density (ODc) value defined as three standard deviation values above the mean OD of the negative control.

$OD \leq 2 \times ODc$ non-adherent/non-biofilm producer (0);
$ODc < OD \leq 2 \times ODc$ weakly adherent/weak biofilm producer (+);
$2 \times ODc < OD \leq 4 \times ODc$ moderately adherent/moderate biofilm producer (++);
$4 \times ODc < OD$ strongly adherent/strong biofilm producer (+++).

3.14 STATIC MONOCOMPARTMENTAL MODELS FOR SIMULATED GASTROINTESTINAL CONDITIONS

The gastrointestinal environment conditions was simulated based on previous studies (Abadía-Garcia et al. 2013; Guerra et al. 2012).

Initially the single strains were added ($OD_{600\ nm}$ =0.04) to artificial saliva medium (6.2 g/ L NaCl, 2.2 g/ L KCl, 0.22 g/ L $CaCl_2$ and 1.2 g/ L $NaHCO_3$) (Abadía-García *et al.*, 2013), in volume for artificial saliva of 1 (saliva):8 (artificial gastrointestinal medium) ratio. The batch culture were maintained 1 minute at 37 ºC and pH 7, through addition of acid (5% (v/v) HCl) or base (5% (w/v) NaOH) (Guerra *et al.*, 2012).

In addition, the mixture was added to a batch culture (2 L maximum capacity) with a magnetic anchor that allowed complete mixing. The batch culture contained TSB (Merck, Germany) and 3 g/ L of pepsin from porcine gastric mucosa (Pranreac AppliChem, Spain) (artificial gastrointestinal medium). Medium pH was adjusted to 4.

Samples of the mixture were withdrawn at 37 ºC and the motion drop method on TSA (Merck, Germany) was used to count the CFU (Guerra *et al.*, 2012, Abadía-García *et al.*, 2013). Plates of TSA were after incubated for 24 hours at 37 ºC. This procedure was performed in triplicate.

3.15 PLANKTONIC GROWTH CURVES

Growth curves were determined for the strains that showed ability to survive under simulated human gastrointestinal conditions. The growth data was determined using a FLUOstarOPTIMA (BMGLabtech, U. K.).

Single species suspensions (optical density at 610 nm was adjusted to 0.4) were dispensed into sterile 96-well microplates. Every 30 minutes reading of absorbance of each well (scanned at 600 nm) in the microplates was monitored over 48 h time period and the results were recorded automatically at 37 ºC.

At least three independent experiments were performed. Data were analysed using Excel 2013 to calculate means and standard deviation for each strain.

3.16 INVASION OF HUMAN CELLS

3.16.1 Preparation of strains

Overnight bacterial cultures (8 -16 h) in 10 mL grown in BHI broth at 30 °C with shaking at 150 rpm were wash three times in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen, USA) by alternate rounds of centrifugation at room temperature (3772 *g*, 10 min). Afterwards culture supernatants were removed and bacterial pellets were ressuspended in an equivalent volume of DMEM, to obtain an $OD_{600\ nm}$ of 0.5.

3.16.2 Human colon adenocarcinoma cells line (Caco-2) and culture condition

The human colon adenocarcinoma cells line Caco-2 was obtained from Deutsche Sammulung von Mikroorganismen and Zellkulturen (Braunschweig, Germany) and was used between passage numbers 20-25. The cells were maintained in essential medium containing glucose (5.55 mM) and supplemented with fetal calf serum (FCS, 15%) at 37 ºC in a humidified atmosphere of 5% CO_2 - 95% air and were grown in minimum, 25 mM HEPES, 100 U/ mL penicillin, 100 µg/ mL streptomycin, and 0.25 µg/ mL amphotericin B. Culture medium was changed every 2 to 3 days and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin - EDTA, 5 min, 37 °C), split 1:3, and subcultured in plastic culture dishes (21 cm^2; Ø = 60 mm; Corning Costar, Corning, NY, USA). For experiments, Caco-2 cells were seeded on 24-well plastic cell culture clusters (1.9 cm^2; Ø = 15.4 mm; TPP®, Trasadingen, Switzerland) with coverslips.

Fine forceps were needed to move the coverslips to 24-well microplates. Coverslips were placed with the shiny surface facing down to allow cell attachment to the opaque surface.

3.16.3 Invasion bioassay (attached and internalized cells)

Infection of Caco-2 cells with the selected bacteria was carried out essentially as described by Edwards and Massey (2011), with small changes. Strains attachment/internalization to human colon adenocarcinoma cells line Caco-2 was assayed in 24-well microplates. Ten µL of washed bacterial suspension ($OD_{600\ nm}$=0.5) were added to each well containing a washed coverslip with 50% of the confluent layer of Caco-2 cells, with 490 µL of DMEM supplemented with 15% FCS. The microplates were incubated for 1 h at 37 ºC in a humidified atmosphere of 5% CO_2. To determine the total number of bacterial cells associated with the Caco-2 cells (attached and internalised), the coverslips were plunged three times in PBS and transferred to new wells containing 500 µL 0.5% Triton X-100 in PBS. The determination of the number of bacterial cells (attached and internalised) was done through the counting of CFU. For CFU counts, cells were diluted serially in saline water (0.85 NaCl) and plated on TSA which were incubated for 24 h at 30 ºC.

To quantify only the number of internalised bacteria cells, the coverslips were removed to new 24-well microplates containing 500 µL DMEM/15% FCS supplemented with 60 µg/ mL imipenem (IPM). The microplates were incubated at 37 ºC in 5% CO_2 atmosphere for 1 h, in order to kill all extracellular bacteria (attached cells). Afterwards, the coverslips were washed 3 times in PBS, the cells were lysed and the number of CFU were determined by plating on TSA as described for the attached/internalised cells.

3.17 FLUORESCENCE MICROSCOPY

For fluorescence microscopy, round coverslips were placed in the wells of 24-well culture plates, as described above in item 3.16. Afterwards, coverslips were removed, and the cells were fixed with paraformaldehyde (3.7% in PBS, 15 minutes at room temperature) (Merck, Germany) and washed three time with PBS at room temperature. Caco-2 cells were permeabilized (10 minutes at room temperature) with 0.2% (V/V) Triton X-100 (Sigma, Portugal) in PBS under stirring (50 rpm) in an orbital shaker, and then incubated with the 1% (w/v) BSA solution in PBS (30 min). After this time the samples were incubated with Alexa Fluor 488 phalloidin (maximum excitation: 495 nm, maximum emission: 519 nm)

(Thermo Fisher, Portugal) diluted 1:100 in the BSA/PBS solution (20 min) and them washed with PBS. After this step the samples were incubated with DAPI (diluted 1:10 000 in PBS - final DAPI concentration: 0.1 µg/ mL) at room temperature for 10 minutes. Finally the samples were extensively washed with PBS and two drops of Fluoromount™ (Sigma, Portugal) were added. All these steps were performance under stirring (50 rpm) in an orbital shaker (IKA KS 130 Basic, USA).

3.18 STATISTICAL ANALYSIS

Data were analyzed by analysis of variance (ANOVA) using the statistical program SPSS version 22.0 (Statistical Package for the Social Sciences). Statistical calculations were based on a confidence level ≥ 95% ($P < 0.05$ was considered statistically significant).

CHAPTER 4. OCCURRENCE AND PHYLOGENETIC DIVERSITY OF ANTIBIOTIC RESISTANT *AEROMONAS* SPP. AMONG WILD ANIMALS

DIAS, C., SERRA, C.R., SIMÕES, L.C., SIMÕES, M., MARTINEZ-MÚRCIA, A., SAAVEDRA, M.J. (2014). Extended-spectrum *β*-lactamase and carbapenemases-producing *Aeromonas* spp. in wild animals from Portugal. *Veterinary Record*, 174:532. (doi: 10.1136/vr.101803)

ABSTRACT

The genus *Aeromonas* is receiving increasing attention as a disease-causing pathogen of animals and as an emerging player in human infectious diseases, such as gastroenteritis or wound infections. Accordingly, the incidence and spread of *Aeromonas* species resistant to different classes of antibiotics is a growing concern. In this study, faeces of wild animals were collected in order to assess *Aeromonas* spp. occurrence and phylogenetic diversity. On the basis of *gyr*B sequence alignments it was observed that the strains clustered in 4 phylogenetic groups with 9 *gyr*B different sequences: *A. salmonicida*, *A. eucrenophila*, *A. bestiarum* and *A. veronii*. Antibiotic susceptibility profiles were established and strains were screened for the presence of different antibiotic resistance genes, using PCR amplification. The Aeromonads collection was less susceptible to penicillins, first generation cephalosporins, erythromycin and tetracycline, reinforcing the utility of extended spectrum-cephalosporins and quinolones as therapeutic options in *Aeromonas* infections. Resistance to *β*-lactam antibiotics correlated with the presence of several *β*-lactamases encoding genes: eleven encoding sequences of OXA-aer, nine FOX and seven CphA genes were found and no TEM-, SHV-, IMP- or VIM-types were identified. Wild animals are a reservoir of antibiotic resistant *Aeromonas* spp. and might represent important vehicles of dissemination of these pathogens.

Keywords: *Aeromonas* spp., antibiotic resistance, *β*-lactamases, wild animals

4.1 INTRODUCTION

The genus *Aeromonas* is receiving increasing attention from the scientific, veterinary and medical fields as a disease-causing pathogen of aquatic and terrestrial animals, but also as an emerging player in human infectious diseases such as gastroenteritis, septicemia, respiratory or wound infections (Jandaand Abbott, 2010, Kaoand Kao, 2011, Liakopoulos *et al.*, 2011, Nagata *et al.*, 2011). *Aeromonas* are Gram-negative, facultative-anaerobic, non-spore-forming, glucose-fermenting, oxidase- and catalase-positive rods (Martin-Carnahanand Joseph, 2005). Species of this genus are known to be ubiquitous in several habitats, mainly in aquatic environments (Jandaand Abbott, 2010). Apart from fish, which are widely reported receptacles for Aeromonads, insects, crustaceans, molluscs, amphibians, reptiles, birds and mammals were also found to harbour *Aeromonas* species, both in healthy and disease state (Pearson *et al.*, 2000, Turutoglu *et al.*, 2005, Vaseeharan *et al.*, 2005, Ottaviani *et al.*, 2006). Regularly, *Aeromonas* species are also components of the microbial communities associated to humans. They are recovered from multiple human samples (tissues, faeces, blood and other fluids) usually as causative agents of infection, associated or not with other microorganisms (Demarta *et al.*, 2000, Hua *et al.*, 2004, Hiransuthikul *et al.*, 2005, Galindo *et al.*, 2006, von Graevenitz, 2007).

The susceptibility of *Aeromonas* species to traditional antibiotic treatments is not well documented, and cases of resistance to different classes of antibiotics, including chloramphenicol, tetracyclines, sulfonamides, quinolones, penicillins and cephalosporins have been reported over the last 20 years (Jandaand Abbott, 2010). An increase in resistance levels of the genus *Aeromonas*, particularly to β-lactam antibiotics, has been observed not only in clinical strains, but also in environmental strains (Saavedra *et al.*, 2004, Saavedra *et al.*, 2007). The complete genome sequencing of *A. hydrophila* ATCC7699 and *A. salmonicida* A449 revealed that both strains carry an array of genes to counteract antibacterial factors present in the environment, including several antibiotics used for human and animal clinical treatment (Seshadri *et al.*, 2006, Reith *et al.*, 2008).

The most popular mechanism of antibacterial resistance among Aeromonads is the production of three chromosomally encoded, which have been described and identified in different *Aeromonas* species (Jandaand Abbott, 2010).

The *β*-lactamases may or not concomitantly occur in the same strain and their coordinated expression is induced by the presence of *β*-lactam antibiotics (Walsh *et al.*, 1995, Alksneand Rasmussen, 1997, Walsh *et al.*, 1997, Avison *et al.*, 2004). These enzymes comprise molecular cephalosporinases (Bush-Jacoby-Medeiros group 1 or Ambler's class C), penicillinases/oxacillinases such as OXA-type enzymes (Bush-Jacoby-Medeiros group 2d or Ambler's class D) and metallo-*β*-lactamases (Bush-Jacoby-Medeiros group 3 or Ambler's class B) (Hayes *et al.*, 1994, Walsh *et al.*, 1995, Walsh *et al.*, 1997). Much less frequently, extended-spectrum *β*-lactamases (ESBLs) such as TEM-type, SHV-type and PER-type enzymes (class A *β*-lactamases), have also been identified in sporadic cases involving Aeromonads (Fosse *et al.*, 2004, Libisch *et al.*, 2008, Picão *et al.*, 2008). The most common class B *β*-lactamases produced by this genus are of the "CphA" type, whose sequences appear to be widely distributed in *A. hydrophila* an *A. veronii* strains (Walsh *et al.*, 1997). Recently, two other metallo-*β*-lactamases, VIM and IMP, have been identified in *A. hydrophila* and *A. caviae*, encoded on an integrons and a plasmid, respectively (Neuwirth *et al.*, 2007, Libisch *et al.*, 2008).

This study reports the isolation and identification of *Aeromonas* spp. from wild animals, the antibiotic resistance found in the strains isolated and the association of resistance with the presence of bla_{CphA}, bla_{IMP}, bla_{VIM}, bla_{OXA}, bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{MOX} and bla_{FOX} genes in the strains of the isolated *Aeromonas* spp..

4.2 MATERIAL AND METHODS

4.2.1 Sample collection, bacterial isolation and growth conditions

The fecal samples from different wild animal species were collected and isolated according to the scheme previously stated in sub-section 3.1. In this study, colonies that were morphologically suspected as *Aeromonas* spp. (yellow, smooth and round) were then selected to establish pure cultures. Direct streaking of fecal swabs on glutamate starch phenol-red (GSP) agar medium (Merck) was used to select, strain and purify bacterial strains by incubating plates aerobically at 30 ºC for 24 h. All strains were checked for oxidase (1% tetramethylphenylenediamine, Merck) activity before cryopreservation in BHI with 15% glycerol at -70 ºC.

4.2.2 Identification of strains

4.2.2.1 API 20 NE system test

The biochemical profile of bacterial strains was determined using the standard classification technique API 20NE (BioMérieux, Marcy l'Etoile, France), following the manufacturer's instructions (Sub-section 3.2.1).

4.2.2.2 DNA extraction, PCR amplification, DNA sequencing and phylogenetic analysis

Total genomic DNA was extracted from overnight pure colonies as previously described (Soler *et al.*, 2004). Oligonucleotide primers used for amplification of *gyr*B and *β*-lactamase encoding genes are listed in Table 3.1 sub-section 3.2.2.2 (Yáñez *et al.*, 2003, Saavedraand Martínez-Murcia, 2005, Fontes, 2009). Detection of *β*-lactamase encoding genes was done for TEM-type, SHV-type, CTX-M-type, MOX and FOX variants of extended-spectrum-*β*-lactamases (ESBLs) and for the CphA, IMP-type, VIM-type and OXA-type carbapenemases.

PCR reaction mixtures (50 μL) contained 5 μL of Biotools PCR buffer (10×), 200 μM of each nucleotide, 10 pmol of each primer, 1 μL (1U) of *taq* DNA polymerase (Biotools) and 1 μL of template genomic DNA.

The conditions for gene amplification were: initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles composed of a denaturation step at 94 °C for 15 seconds, an annealing step at 55 °C for 30 seconds (except for bla_{FOX}, in which 60 ºC were used) and an extension period at 72 °C for 45 seconds. Finally, the mixture underwent a cycle of final extension at 72 °C for 3 minutes.

Sequencing of 16S rRNA and *gyr*B genes was performed as previously described (Martínez-Murcia *et al.*, 1999, Soler *et al.*, 2004), except for the BigDye Terminator V3.1 Cycle Sequencing Kit in the ABI 3100-Avant Genetic Analyzer (Applied Biosystems), which was according to the manufacturer's instructions.

Newly determined sequences were aligned to previously published reference sequences (Martínez-Murcia *et al.*, 1992, Martínez-Murcia *et al.*, 1999, Yáñez *et al.*, 2003, Soler *et al.*, 2004, Saavedra *et al.*, 2006) by the Clustal X alignment program, version 1.8 (Thompson *et al.*, 1997).

Genetic distances were obtained by Kimura's 2 parameter model (Kimura, 1980) and the evolutionary tree was constructed by the Neighbour-Joining method (Saitouand Nei, 1987) with the Molecular Evolutionary Genetics Analysis (MEGA) program, version 2.1 (Kumar *et al.,* 2001)(Sub-section 3.2.2).

4.2.3 Antimicrobial drug susceptibility testing

The antimicrobial drug susceptibility of *Aeromonas* strains was determined by the disk method of Kirby-Bauer (Bauer *et al.,* 1966) on MHA plates (Oxoid) according to the CLSI (2006), and the procedure described in sub-section 3.3.1. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced.

4.3 RESULTS

4.3.1 Prevalence of *Aeromonas* in wild animals

A total of 325 bacterial strains were obtained from the 140 fecal samples of wild animals analysed in this survey (Table 3.1, Sub-section 3.1), with 8 fecal samples (5%) positive for the presence of *Aeromonas* spp.. Plating of fecal swabs in GSP (a selective and differential medium for *Pseudomonas* spp. and *Aeromonas* spp.) originated the appearance of 16 yellow, smooth and round colonies, putative *Aeromonas* spp., in 4 samples from *Cervus elaphus* (red deer), 1 sample of a *Strix aluco* (tawny owl), 1 sample of a *Sciurus vulgaris* (red squirrel), 1 sample of a *Colubridae* (snake) and 1 sample of a *Circaetus gallicus* (short-toed snake eagle) (Table 4.1).

All 16 strains tested were positive for oxidase activity, a characteristic of *Aeromonas* species (Abbott *et al.*, 2003). Phylogenetic classification and species identification was achieved by sequencing the housekeeping gene *gyr*B that encodes the *β*-subunit of the type II DNA topoisomerase, DNA gyrase (Table 4.1).

Ten different *gyr*B gene partial sequences were obtained that grouped *Aeromonas* strains into four different species: *A. salmonicida* (n=9), *A. eucrenophila* (n=4), *A. bestiarum* (n=2) and *A. veronii* (n=1) (Figure 4.1).

Table 4.1 Identification of bacterial strains

Isolate nº	Animal of origin	API20 NE		*gyr*B sequence analysis
		Taxon	%ID	Closest known species
AS006C3b1	*Cervus elaphus* (red deer)	*A. hydrophila*	97.6	*A. salmonicida*
AS006C3c1	*Cervus elaphus* (red deer)	*A. hydrophila*	97.6	*A. salmonicida*
AS006C3c2	*Cervus elaphus* (red deer)	*A. sobria*	64.5	*A. salmonicida*
AS006C3d1	*Cervus elaphus* (red deer)	*A. sobria*	64.5	*A. salmonicida*
AS017A3a	*Strix aluco* (tawny owl)	*A. hydrophila*	99.8	*A. salmonicida*
AS036A2a1B	*Sciurus vulgaris* (red squirrel)	*A. hydrophila*	98.7	*A. bestiarum*
AS036B1a2	*Sciurus vulgaris* (red squirrel)	*A. hydrophila*	86.9	*A. bestiarum*
AS044A2a1(A)	*Colubridae* (snake)	*A. caviae*	97.6	*A. eucrenophila*
AS044A2a1(B)	*Colubridae* (snake)	*A. caviae*	97.6	*A. eucrenophila*
AS044A2a1(C)	*Colubridae* (snake)	*A. caviae*	96.8	*A. eucrenophila*
AS044B2a1A	*Colubridae* (snake)	*A. caviae*	97.6	*A. eucrenophila*
AS070GSP1	*Circaetus gallicus* (short-toed snake eagle)	*A. sobria*	96.8	*A. veronii*
AS115A2GSP1	*Cervus elaphus* (red deer)	*A.hydrophila*	99.0	*A. salmonicida*
AS117GS2A	*Cervus elaphus* (red deer)	*A. hydrophila / A.caviae*	78.0	*A. salmonicida*
AS117GSP2c1	*Cervus elaphus* (red deer)	*A. hydrophila / A.caviae*	98.7	*A. salmonicida*
AS119GSP1c1	*Cervus elaphus* (red deer)	*A. sobria*	64.5	*A. salmonicida*

The unrooted phylogenetic tree was constructed by using the *gyr*B gene partial sequence of each strain and previously published reference sequences (Martínez-Murcia *et al.*, 1992, Martínez-Murcia *et al.*, 1999, Yáñez *et al.*, 2003, Soler *et al.*, 2004, Saavedra *et al.*, 2006) strains from the Molecular Diagnostics Center (MDC, Biomolecular Technologies S.L.U., Alicante, Spain) culture collection, including type strains (Figure 4.1). The sequencing results did not, however, coincide with the identification obtained when using the biochemical classification method API 20NE (Table 4.1).

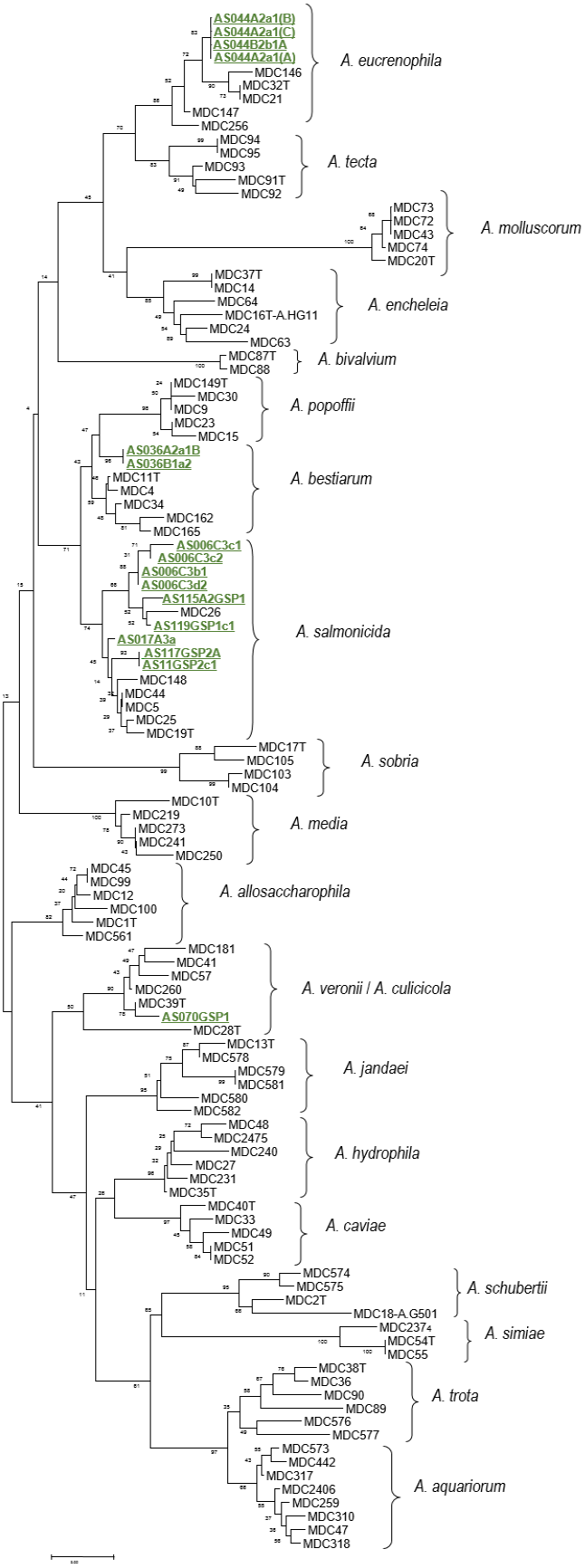

Figure 4.1 Unrooted phylogenetic tree based on *gyr*B gene sequences of strains isolated in this study and the representative strains of the genus *Aeromonas* of the MDC collection.

4.3.2 Susceptibility of *Aeromonas* strains to antibiotics

The antimicrobial resistance profile of *Aeromonas* strains was established by the disk method of Kirby-Bauer (Bauer *et al.*, 1966). The patterns of resistance to 27 antibiotics belonging to different classes are summarized in Table 4.2. *β*-lactam antibiotics tested included penicillins (aminopenicillins, carboxypenicillins and ureidopenicillins), cephalosporins (1st, 3rd, and 4th generations), monobactams and carbapenems. Additionally, antibiotics belonging to other classes were also tested. Those included quinolones (nalidixic acid and ciprofloxacin), aminoglycosides (amikacin, gentamicin, tobramycin, kanamycin and streptomycin), macrolides (erythromycin), tetracyclines (tetracycline), chloramphenicol, fosfomycin and the combination sulfamethoxazole/trimethoprim.

Resistance to at least two antibiotics was recorded for all strains, and almost half (43.8%) of strains were found to be multiresistant (resistant to four or more antibiotics) (Table 4.2). Multiresistance was previously reported when analyzing *Aeromonas* spp. isolated from fish (Radu *et al.*, 2003). The present results showing 93.7% of strains resistant to amoxicillin (AML), 81.25% to cephalothin (KF) and 37.5% to ticarcillin (TIC) are in agreement with this report (Table 4.2). The combination of an aminopenicillin and a carboxipenicillin with a *β*-lactamase inhibitor (clavulanic acid), was effective in reducing resistance, as shown by the decrease in the proportion of resistant strains. This reduction was more pronounced with amoxicillin (93.7% *versus* 25%) than with ticarcillin (37.5% *versus* 31%). All the other *β*-lactam antibiotics tested, including the monobactam aztreonam, the carbapenem imipenem and the extended spectrum cephalosporins were efficient against all the strains tested.

Beside penicillins and the narrow spectrum cephalosporin cephalothin (KF; Table 4.2), considerable high resistance rates were recorded for streptomycin, erythromycin and tetracycline (S, E and TE respectively; Table 4.2), as previously reported by other authors (Son *et al.*, 1997). All the other antibiotics tested were effective against the *Aeromonas* strains.

Genes analysed included *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{CTX-M,}}$ *bla*$_{\text{FOX}}$, *bla*$_{\text{MOX}}$, *bla*$_{\text{CphA}}$, *bla*$_{\text{IMP}}$ and *bla*$_{\text{VIM}}$ and the results are present in Table 4.2. A plus (+) symbol indicates that the PCR amplification gave a product of the expected size, suggesting the presence of the corresponding gene.

Table 4.2 Characterisation of the strains isolated concerning resistance phenotype and *bla* genotypes

Strains	Resistance phenotype	*β*-lactamases genes content				
		***bla*$_{OXA-aer}$**	***bla*$_{CTX-M}$**	***bla*$_{FOX}$**	***bla*$_{MOX}$**	***bla*$_{CphA}$**
A. salmonicida $_{AS006C3b1}$	AML, AMC, TIC, TIM, KF, S, E,TE	-	-	-	-	-
A. salmonicida $_{AS006C3c1}$	KF, S, E	-	-	-	-	-
A. salmonicida $_{AS006C3c2}$	AML, AMC, TIC, TIM, KF, S, E, TE	-	-	-	-	+
A. salmonicida $_{AS006C3d1}$	AML, AMC, TIC, TIM, KF, S, E, TE	-	-	+	-	+
A. salmonicida $_{AS017A3a}$	AML, AMC, TIM, PRL, TZP, KF, E, TE	-	-	-	+	+
A. salmonicida $_{AS115A2GSP1}$	AML, KF, E	+	-	-	-	+
A. salmonicida $_{AS117GS2A}$	AML, TIC, KF, S	+	-	+	-	-
A. salmonicida $_{AS117GSP2c1}$	AML, TIC, KF, S	+	-	+	-	-
A. salmonicida $_{AS119GSP1c1}$	AML, KF	+	-	+	-	-
A. bestiarum $_{AS036A2a1B}$	AML, E	+	-	-	-	+
A. bestiarum $_{AS036B1a2}$	AML, E	+	-	-	-	+
A. eucrenophila $_{AS044A2a1(A)}$	AML, KF, E	+	-	+	-	-
A. eucrenophila $_{AS044A2a1(B)}$	AML, KF, E	+	-	+	-	-
A. eucrenophila $_{AS044A2a1(C)}$	AML, KF, E	+	-	+	-	-
A. eucrenophila $_{AS044B2a1A}$	AML, KF, E	+	-	+	-	-
A. veronii $_{AS070GSP1}$	AML, TIC, TIM, NA, S, E, TE	+	+	+	+	+

The antibiotic resistance genetic determinants studied were present in fourteen strains (88%), with thirteen strains (81%) carrying more than one of the genes studied. The *β*-lactamases genes were found in different combinations, and in one case, five *β*-lactamase genes were detected in the same strain, *A. veronii* $_{AS070GSP1}$. The most prevalent genes were *bla*$_{OXAaer}$ present in eleven strains (69%) followed by *bla*$_{FOX}$ detected in nine strains (56%) and the metallo-*β*-lactamase encoding gene *bla*$_{cphA}$, detected in seven strains (44%). The gene *bla*$_{MOX}$ was detected in two strains (*A. salmonicida* $_{AS017A3a}$, *A. veronii* $_{AS070GSP1}$) and *bla*$_{CTX-M}$ in *A. veronii* $_{AS070GSP1}$ strain.

Finally, no PCR specific for *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-B}$, *bla*$_{OXA-C}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ encoding sequences were detected, yielding no evidence for the presence of these genes in any strain.

4.4 DISCUSSION

This work aimed to investigate the occurrence and phylogenetic diversity of *Aeromonas* spp. a recognised pathogen, in wild animal from the north of Portugal. A total of 16 strains of *Aeromonas* were collected, and phylogenetic analysis revealed 10 different *gyr*B sequencing, grouped *Aeromonas* strains into four different species (*A. salmonicida, A. eucrenophila, A. bestiarum* and *A. veronii*).This gene has been demonstrated to be an excellent molecular chronometer for phylogenetic inference in the genus *Aeromonas*, being considered much more accurate than the commonly used 16S rRNA gene sequencing (Jandaand Abbott, 2010). Discrepancy between both methods of identification has previously been reported (Jandaand Abbott, 2010). In such cases, studying two or more housekeeping genes, improves the phylogenies reliability and differentiation power (Soler *et al.*, 2004).

Since complete identification was beyond the objective of this study, no additional identification methods, such as sequencing *rpoD* (the σ^{70} sigma factor encoding gene) were employed. It is, however, important to highlight the fact that clinically significant species, responsible for severe human infections (i.e., *A. hydrophila sensu stricto*, *A. caviae sensu stricto* an *A. veronii*) might be present among the isolated strains.

In this study, antibiotic susceptibility patterns and genetic bases of antibiotic resistance among the 16 bacterial strains were also assessed. *Aeromonas* strains from different sources have been reported to have a relatively high resistance to *β*-lactam antibiotics, usually correlated with naturally occurring phenotypes of *β*-lactamases production (Fosse *et al.*, 2003).Tetracycline and erythromycin resistance are among the most widespread form of antibiotic resistance in the bacterial kingdom. Their intensive (ab)use as growth promoters in animal husbandry during the last decades, as well as therapeutic and prophylactic agents, together with the association of their genetic determinants with mobile elements, like plasmids or conjugative transposons, has led to a worldwide increase in the frequency of resistant bacterial species (Enne, 2010). Commonly, the recommended first therapeutic options for *Aeromonas* infections are fluoroquinolones (Parkerand Shaw, 2011). Although the majority of strains were susceptible to the quinolones tested, confirming the utility of such antibiotics in the treatment of *Aeromonas* infections, one strain, *A. veronii* AS070GSP1, was resistant to nalidixic-acid (NA; Table 4.2). Quinolone-resistant strains of *A. hydrophila*, *A. caviae*, *A. media, A. salmonicida, A. popoffii* and *A. veronii*, recovered from humans, freshwater and eels, were previously reported (Alcaide *et al.*, 2010, Arias *et al.*, 2010). In particular, strains of *A. caviae* and *A. veronii* isolated from stools of patients with gastrointestinal symptoms

were resistant to nalidixic-acid (Arias *et al.*, 2010). Resistance in such strains was due to point mutations at the quinolone targets *gyrA* and *parC* genes and not due to quinolone efflux pumps or plasmid mediated quinolone resistance determinants (Alcaide *et al.*, 2010, Arias *et al.*, 2010). It is expectable that the observed resistance in the *A. veronii* $_{AS070GSP1}$ is also derived from mutations in such genes.

Inducible chromosomal *β*-lactamases is the resistance mechanism against *β*-lactam antibiotics for most Aeromonads, with examples described and identified in different *Aeromonas* species (Walsh *et al.*, 1995, Walsh *et al.*, 1997, Henriques *et al.*, 2006). Expression of metallo-*β*-lactamases active against carbapenems is also a concern with regards to *Aeromonas* infections (Jandaand Abbott, 2010, Parkerand Shaw, 2011). In light of this knowledge, all *Aeromonas* strains of this study were screened by PCR for the presence of *β*-lactamase genes. The OXA-aer, FOX and CphA were the detected at a higher extent. The "CphA" type is considered the most common metallo-*β*-lactamase produced by *Aeromonas* species (Jandaand Abbott, 2010), corroborating the present results. The *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-B}$, *bla*$_{OXA-C}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ genes were not found in the studied strains. Few *bla* genes have been reported in aeromonads other than the genes codifying for the mentioned chromosomal penicillinases, cephalosporinases and carbapenemases. SHV, TEM and OXA narrow spectrum enzymes have been rarely encountered in these specimens (Henriques *et al.*, 2006, Picão *et al.*, 2008) as well as extended-spectrum *β*-lactamases (Picão *et al.*, 2008) and VIM and IMP metallo-*β*-lactamases acquired enzymes were identified in *Aeromonas* in only one occasion (Neuwirth *et al.*, 2007, Libisch *et al.*, 2008).

4.5 CONCLUSION

Several antibiotic resistant *Aeromonas* species were recovered from the faeces of wild animals. The majority of strains were resistant to penicillin and narrow-spectrum cephalosporins, and susceptible to extended spectrum-cephalosporins and quinolones, reinforcing the utility of such antibiotics as therapeutic options in *Aeromonas* infections. Resistance to *β*-lactam antibiotics correlates with the presence of several *β*-lactamases encoding genes, including the “CphA” type metallo-*β*-lactamases, in the genome of the strains tested. One strain of *A. veronii* $_{AS070GSP1}$, isolated from an eagle, was resistant to nalidixic acid and had a combination of five different *β*-lactamases encoding genes. Birds, such as eagles, with their migrating behaviour, might represent important vehicles of dissemination of these pathogens.

CHAPTER 5. ADHESION, BIOFILM FORMATION AND MULTIRESISTANT *AEROMONAS* SPP. FROM WILD ANIMALS

DIAS, C., BORGES, A., SAAVEDRA, M.J., SIMÕES, M. (2017). Biofilm formation and multidrug resistant *Aeromonas* spp. from wild animals. *Journal of Global Antimicrobial Resistance.* (JGAR-D-17-00303). *In press* (http://dx.doi.org/10.1016/j.jgar.2017.09.010).

ABSTRACT

The "One Health" concept recognizes that the health of humans, animals and the environment are interconnected. Therefore, the knowledge on the behavior of microorganisms from the most diverse environmental niches is important to prevent emergence and dissemination of antimicrobial resistance. Wild animals are known to carry antimicrobial resistant microorganisms with potential public health impact. However, no data is available on the sessile behavior of bacteria from wild animals, even if antimicrobial resistance is amplified in biofilms. This study characterizes the adhesion and biofilm formation abilities of 14 distinct *Aeromonas* strains (eight *A. salmonicida*, three *A. eucrenophila*, two *A. bestiarum* and one *A. veronii*) isolated from the faeces of wild animals and previously characterized as resistant to β-lactam antibiotics. Their susceptibility to the antibiotic ciprofloxacin (CIP) was tested against planktonic bacteria and biofilms. All the *Aeromonas* strains had hydrophilic surfaces and were able to adhere and form biofilms with distinct magnitudes. All the strains were resistant to CIP. Biofilm exposure to 10 times the minimum inhibitory concentration of CIP caused low to moderate removal. The results of this study propose that the strains tested are of potential public health concern and emphasize that wild animals are potential reservoirs of multiresistant strains. In fact, *Aeromonas* species are consistently considered opportunistic pathogens. The bacterial ability to form biofilms increases antimicrobial resistance and the propensity to cause persistent infections.

Keywords: *Aeromonas*, biofilm formation, biofilm susceptibility, multiresistant, wild animals

5.1 INTRODUCTION

The growth in the human population have prompted the encroachment of human into new environments and resulted in environmental disrupting of habitats, bringing human and domestic animals into contact with wildlife (Pesapane *et al.*, 2013, Gortazar *et al.*, 2014). Close contact provides more opportunities for diseases to pass between animals and people (Martin *et al.*, 2011). Most of emerging infectious diseases in people are spread from animals (Openshaw *et al.*, 2016, Reperant *et al.*, 2016). Those infections are problematic as they can re-emerge after they are considered to be under control or eradicated. In many industrialized countries, diseases caused by antibiotic resistant bacteria are of particular importance for at-risk groups such as old, young, and immunocompromised individuals (Cantasand Suer, 2014).

Surprisingly, new zoonotic diseases have been caused by opportunistic pathogens arising from wild animal reservoirs (Blancou *et al.*, 2005). This clearly proposes that different ecosystems are inextricably connected. In this context, the "One Health" initiative proposes a global strategy developed for expanding interdisciplinary collaborations in all aspects of health care for humans, animals and the environment. The main aim is to achieve the best health (Wallace *et al.*, 2013, Gibbs, 2014).

Aeromonas form a complex genus of Gram-negative bacteria that can be found in most aquatic environments, including drinking water (Yáñez *et al.*, 2003). In the last years, *Aeromonas* spp. have received increasing attention as opportunistic pathogens (Vilches *et al.*, 2004, Sha *et al.*, 2005, Yu *et al.*, 2005, Jandaand Abbott, 2010). They are important pathogens for fish and reptiles, and can occasionally cause disease in other animals and in humans (Pearson *et al.*, 2000, Turutoglu *et al.*, 2005, Evangelista-Barreto *et al.*, 2006, Ceylan *et al.*, 2009). *Aeromonas*-related infections are of particular concern in immunocompetent and immunocompromised hosts (Martin-Carnahanand Joseph, 2005, Parkerand Shaw, 2011), with systematic and localized manifestations like septicemia, wound infections, meningitis, endocarditis, and gastroenteritis (Biscardi *et al.*, 2002, Jandaand Abbott, 2010). The pathogenesis of *Aeromonas* species includes an extensive group of virulence determinants, many of them similar to virulence factors found in human pathogens (Chopra *et al.*, 2009). Virulence mechanisms of *Aeromonas* are complex and poorly understood. Virulence-associated determinants include lipopolysaccharides, the S-layer outer-membrane protein, pili and flagella, resistance to complete-mediated lysis and type III secretion system (Turutoglu *et al.*, 2005, Ceylan *et al.*, 2009). Besides their pathogenicity, a visible increase in resistance to

antibiotics, including to *β*-lactams, has been observed not only from clinical strains but also from environmental strains (Saavedra *et al.*, 2004, Saavedra *et al.*, 2007, Varela *et al.*, 2016). *Aeromonas* species are capable to adhere on biotic or abiotic surfaces and form biofilms (Bomo *et al.*, 2004, Scoaris Dde *et al.*, 2008, Chungand Okabe, 2009). Bacterial biofilms are structured clusters of cells enclosed in a self- matrix of extracellular polymeric substance that are attached to a surface (Hosseinidoust *et al.*, 2013). The biofilm lifestyle provides several advantages over growing planktonically, particularly increased resistance to antibiotics (Gilbert *et al.*, 2002).

This work was performed to characterize the antimicrobial susceptibility, adhesion and biofilm formation abilities of 14 diverse *Aeromonas* strains isolated from wild animals, providing insights into their virulence potential, environmental persistence and resilience.

5.2 MATERIAL AND METHODS

5.2.1 Bacterial identification and growth conditions

The bacteria used in this work were obtained from faeces of wild animals (Table 3.1): four samples from *Cervus elaphus* (red deer) (*A. salmonicida* AS006C3b1, *A. salmonicida* AS006C3c1, *A. salmonicida* AS006C3c2, *A. salmonicida* AS115A2GSP1, *A. salmonicida* AS117GSP2A, *A. salmonicida* AS117GSP2C1 and *A. salmonicida* AS119GSP1C1), one from *Strix aluco* (tawny owl) (*A. salmonicida* AS017A3a), one from *Sciurus vulgaris* (red squirrel) (*A. bestiarum* AS036A2a1B and *A. bestiarum* AS036B1a1), one from *Colubridae* (snake) (*A. eucrenophila* AS044A2a1(A), *A. eucrenophila* AS044A2a1(B) and *A. eucrenophila* AS044A2a1(C)) and one sample from *Circaetus gallicus* (short-toed snake eagle) (*A. veronii* AS070GSP1), as described in sub-section 3.1.

5.2.2 Substratum

PS was selected for the adhesion tests. In order to prepare PS, coupons with dimension of 1 cm × 1cm were immersed in a solution of 5% (v/v) commercial detergent (Sonasol Pril, Henkel Ibérica S. A.) and in ultrapure water for 30 min with gentle shaking, according to the procedure described in sub-section 3.9.1.

5.2.3 Determination of bacterial contact angles and surface hydrophobicity

Contact angles were assessed according to Simões et al. (2010) using a model OCA 15 Plus (DATAPHYSICS, Germany) video based optical contact angle measure instrument, allowing image acquisition and data analysis. Hydrophobicity was evaluated from contact angles values, as described by van Oss et al. (1987, 1988, 1989) (Sub-section 3.7).

5.2.4 Determination of minimal inhibitory concentration

The minimal inhibitory concentration (MIC) of ciprofloxacin (CIP) was determined according to the CLSI (2014) guidelines using the broth microdilution method, according to the procedure described in sub-section 3.3.2.

5.2.5 Monolayer adhesion ability on polystyrene

Coupons of PS, prepared as described previously (Sub-section 3.9.2), were placed in the bottom of 12-well (22 mm diameter each well) microtiter plates (polystyrene, Orange Scientific, USA) containing 2 mL of a cell suspension (1×10^8 CFU/ mL in MHB). Initial adhesion to each coupon was allowed to occur by incubating the microtiter plates at 30 ºC for 2 h under 150 rpm agitation. Negative controls were obtained by placing coupons in MHB without bacteria. At the end of the assay, coupons were removed from the wells of the microtiter plates and immersed in 2 mL of sterile saline solution (NaCl, 0.85%) to remove the loosely attached cells. Afterwards the PS coupons were used for biomass quantification by crystal violet (CV) staining. All the assays were done in triplicate with three repeats.

5.2.6 Evaluation of biofilm formation

The biofilm formation assay was done according to a modified microtiter plate method described by Stepanović et al. (2000). Briefly, 200 µL of a standardized cell suspension (1×10^8 CFU/ mL in MHB) was transferred into sixteen wells of a sterile 96-well microtiter plate. The plate was incubated aerobically on a shaker at 150 rpm and 30 ºC, for 24, 48 and 72 h, to allow biofilm development. The medium was carefully discarded and replaced by fresh one on a daily basis (only for biofilms of 48 and 72 h). After each biofilm development period, the content of the wells was removed, and each well was washed three times with

250 µL of sterile saline solution to discard non-adhered cells. The plate was air dried for 30 min, and the remaining attached cells were analysed in terms of biomass adhered on the surface of the microtiter plates. Wells with MHB were used as negative controls. All experiments were repeated three times (Sub-section 3.10).

5.2.7 Biomass quantification

The biomass was quantified by crystal violet (CV) (Gram colour - staining set for microscopy, Merck, Portugal) staining according to Simões et al. (2010). For adhesion assays, PS coupons with adhered bacteria in the 12-wells microtiter plates were removed, immersed in a new plate and the bacteria were fixed with 1 mL of ethanol 98% (v/v) (Vaz Pereira, Portugal). Ethanol was removed after 15 min of contact and the coupons were allowed to dry at room temperature. Afterwards the coupons were stained for 5 min with 600 µL of CV and washed with sterile saline solution, before being immersed in 1 mL of 33% (v/v) acetic acid (Merck, Portugal) to release and dissolve the stain. For biofilm mass quantification, the biofilms in the 96-wells plates were fixed with 250 µl of 98% ethanol per well, for 15 min. Afterwards, the plates were air dried and the dye bound to the biofilm cells was resolubilized with 200 µL of 33% (v/v) glacial acetic acid. The optical density (OD) of each well was measured at 570 nm using an automated microtiter plate reader (BIO-TEK, Model Synergy HT) (Sub-section 3.12).

5.2.8 Adherent/biofilm bacteria classification

Based on OD measurements the strains was classified according to the scheme previously stated in sub-section 3.13. This classification was based upon the cut-off of the optical density (ODc) value defined as three standard deviation values above the mean OD of the negative control Stepanović et al. (2000).

5.2.9 Biofilm control

To determine whether ciprofloxacin had any effect on biofilm control, 24 h-old biofilms formed in 96-well microtiter plates were exposed to 10× MIC according to Simões et al. (2010). Twenty-four hours after exposure the biofilms were analyzed in terms of mass reduction by CV staining. Biofilm removal was given according to equation 8, where

% BR is the percentage of biofilm removal, ODn is the $OD_{570\,nm}$ value of biofilm non-exposed to ciprofloxacin and ODa is de $OD_{570\,nm}$ value for biofilm exposed to the antibiotic. The action of ciprofloxacin on biofilm removal was classified according to Lemos et al. (2014): Removal < 25% - low efficacy; Removal < 60% - moderate efficacy; 60 ≤ Removal < 90% - high efficacy; 90 ≤ Removal ≤ 100% - excellent efficacy.

5.2.10 Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using the statistical program SPSS version 22.0 (Statistical Package for the Social Sciences) (Sub-section 3.16).

5.3 RESULTS

The strains used in this work were obtained from fecal samples of wild animals (Table 5.1): four samples from *Cervus elaphus* (red deer) (*A. salmonicida* AS006C3b1, *A. salmonicida* AS006C3c1, *A. salmonicida* AS006C3c2, *A. salmonicida* AS115A2GSP1, *A. salmonicida* AS117GSP2A, *A. salmonicida* AS117GSP2C1 and *A. salmonicida* AS119GSP1C1), one from *Strix aluco* (tawny owl) (*A. salmonicida* AS017A3a), one from *Sciurus vulgaris* (red squirrel) (*A. bestiarum* AS036A2a1B and *A. bestiarum* AS036B1a1), one from *Colubridae* (snake) (*A. eucrenophila* AS044A2a1(A), *A. eucrenophila* AS044A2a1(B) and *A. eucrenophila* AS044A2a1(C)) and one sample from *Circaetus gallicus* (short-toed snake eagle) (*A. veronii* AS070GSP1).

5.3.1 Hydrophobicity

The surfaces of all *Aeromonas* strains are hydrophilic, $\Delta G_{bwb}^{TOT} > 0$ mJ/ m^2 (Table 5.1). However, the hydrophobicity values were significantly different between some of the strains. Strain *A. eucrenophila* AS044A2a1(C) had the highest surface hydrophobicity value while *A. salmonicida* AS006C3c2 had the lowest value. This difference in hydrophobicity was statistically significant ($P < 0.05$). In the *A. salmonicida* group, of strains it was found that the strains obtained from the same source animal - *Cervus elaphus* (*A. salmonicida* AS006C3b1, *A. salmonicida* AS006C3c1 and *A. salmonicida* AS006C3c2) had significantly different values of hydrophobicity ($P < 0.05$). In the *A. salmonicida* group, three strains isolated from the same animal species, *Cervus elaphus*, showed statistically significant differences in their hydrophobicity values ($P < 0.05$). The six remaining *A. salmonicida* strains (AS006C3c2, AS017A3a, AS115A2GSP1, AS117GSP2A, AS117GSP2C1 and AS119GSP1C1) had similar hydrophobicity values that were

not different from each other ($P > 0.05$). Regarding the remaining physicochemical parameters, the apolar component (γ^{LW}) was in the range of 40 to 42 mJ/ m^2 for *A. salmonicida* strains, with the exception of *A. salmonicida* AS006C3b1 (γ^{LW} = 33 mJ/ m^2).

Table 5.1 Contact angles (in degrees) with water (θ_W), formamide (θ_F), α-bromonaphthalene (θ_B), surface tension parameters (γ^{LW}, γ^{AB}, γ^+, γ^-), free energy of interaction (ΔG_{bwb}^{TOT}) of the strains (b) when immersed in water (w). Values are mean ± SD of three independent experiments

		Contact angle (º)			Surface tension parameters (mJ/ m^2)				Hydrophobicity (mJ/ m^2) ΔG_{bwb}^{TOT}
		θ_W	θ_B	θ_F	γ^{LW}	γ^{AB}	γ^+	γ^-	
Source wild animal	**Strains**								
Cervus elaphus (Red deer)	*A. salmonicida* AS006C3b1	20±2	44±3	14±2	33±0	24±0	3±0	49±0	24±1
	A. salmonicida AS006C3c1	18±1	27±2	20±2	40±0	14±0	1±0	54±0	32±0
	A. salmonicida AS006C3c2	39±6	21±3	29±4	41±0	10±1	0.3±0	37±0	2±1
	A. salmonicida AS115A2GSP1	20±2	46±1	19±1	42±0	6±0	0.2±0	39±1	15±1
	A. salmonicida AS117GSP2A	14±1	39±1	14±1	40±0	13±0	1±0	41±1	16±1
	A. salmonicida AS117GSP2C1	41±2	19±1	34±2	42±0	6±0	0.3±0	38±0	14±0
	A. salmonicida AS119GSP1C1	41±1	17±2	30±3	42±0	8±0	0.5±0	36±0	9±0
Circaetus gallicus (Short-toed snake eagle)	*A. veronii* AS070GSP1	40±2	17±3	34±2	36±0	18±0	2±0	53±0	31±0
Colubridae (Snake)	*A. eucrenophila* AS044A2a1(A)	27±2	37±2	32±1	36±0	12±0	1±0	53±0	34±0
	A. eucrenophila AS044A2a1(B)	23±1	35±1	27±1	37±0	14±0	1±0	54±0	34±1
	A. eucrenophila AS044A2a1(C)	22±7	42±1	31±2	34±0	15±0	1±0	56±1	37±1
Sciurus vulgaris (Red squirrel)	*A. bestiarum* AS036A2a1B	20±2	46±1	19±1	32±0	23±0	3±0	51±0	27±1
	A. bestiarum AS036B1a1	14±1	39±1	14±1	35±0	21±0	2±0	53±0	30±1
Strix aluco (Tawny owl)	*A. salmonicida* AS017A3a	43±3	20±1	35±1	42±0	6 ±1	0.2±0	36±1	10±1
	Substratum								
	PS	83±3	71±2	28±1	39	0.0	0.0	9.9	-44

ΔG_{bwb}^{TOT} < 0 mJ/m^2 - hydrophobic surface; ΔG_{bwb}^{TOT} > 0 mJ/m^2 - hydrophilic surface.

However, this strain had the highest surface polar component (γ^{AB} = 24 mJ/ m^2). *A. salmonicida* AS017A3a had the lowest value for the surface polar component (γ^{AB} = 5.8 mJ/ m^2). *A. bestiarum* AS036A2a1B had the lowest value of apolar component while *A. salmonicida* AS119GSP1C1 had the highest value. All the bacteria had predominantly electron donor surfaces (high γ^- values), with low electron acceptor ability (γ^+). Furthermore, linear regression showed a strong correlation (R^2 = 0.98) between the electron donor parameter and the bacterial surface hydrophobicity (Figure 5.1).

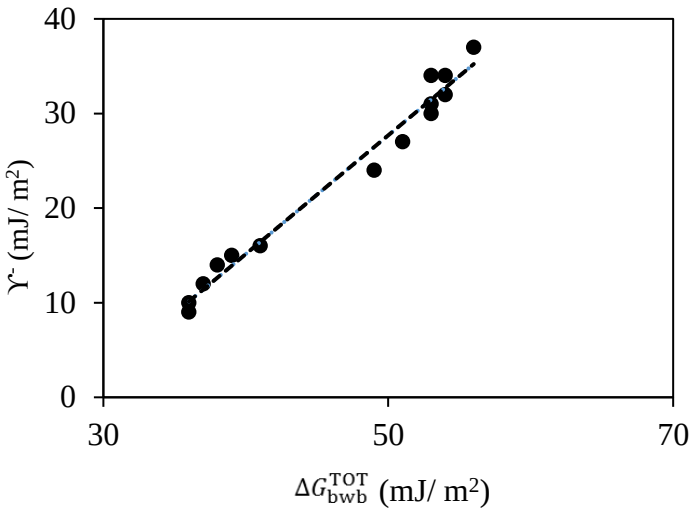

Figure 5.1 Correlation between the electron donor parameter (γ^-) and the cell surface hydrophobicity (ΔG_{bwb}^{TOT}).

5.3.2 Ciprofloxacin (CIP) minimum inhibitory concentration (MIC)

The MIC of CIP was assessed for the diverse *Aeromonas* strains (Table 5.2). The MICs for eight of those strains was 6 µg/ mL. MICs for *A. bestiarum* AS036A2a1B and *A. bestiarum* AS036B1a1 was 24 µg/ mL; while the MIC against *A. salmonicida* AS006C3c2, *A. salmonicida* AS017A3a, *A. salmonicida* AS006C3b1 and *A. salmonicida* AS006C3c1 were 32, 36, 48 and 60 µg/ mL, respectively.

Table 5.2 MIC of ciprofloxacin against the *Aeromonas* spp. strains

Source wild animal	Species	MIC (µg/ mL)
Cervus elaphus (Red deer)	*A. salmonicida* AS006C3b1	48
	A. salmonicida AS006C3c1	60
	A. salmonicida AS006C3c2	32
	A. salmonicida AS115A2GSP1	6
	A. salmonicida AS117GSP2A	6
	A. salmonicida AS117GSP2C1	6
	A. salmonicida AS119GSP1C1	6
Circaetus gallicus (Short-toed snake eagle)	*A. veronii* AS070GSP1	6
Colubridae (Snake)	*A. eucrenophila* AS044A2a1(A)	6
	A. eucrenophila AS044A2a1(B)	6
	A. eucrenophila AS044A2a1(C)	6
Sciurus vulgaris (Red squirrel)	*A. bestiarum* AS036A2a1B	24
	A. bestiarum AS036B1a1	24
Strix aluco (Tawny owl)	*A. salmonicida* AS017A3a	36

5.3.3 Monolayer adhesion ability

Initial bacterial adhesion to PS was also evaluated using a microtiter plate assay and CV staining for biomass assessment (Figure 5.2). The diverse strains showed varying levels of adhesion.

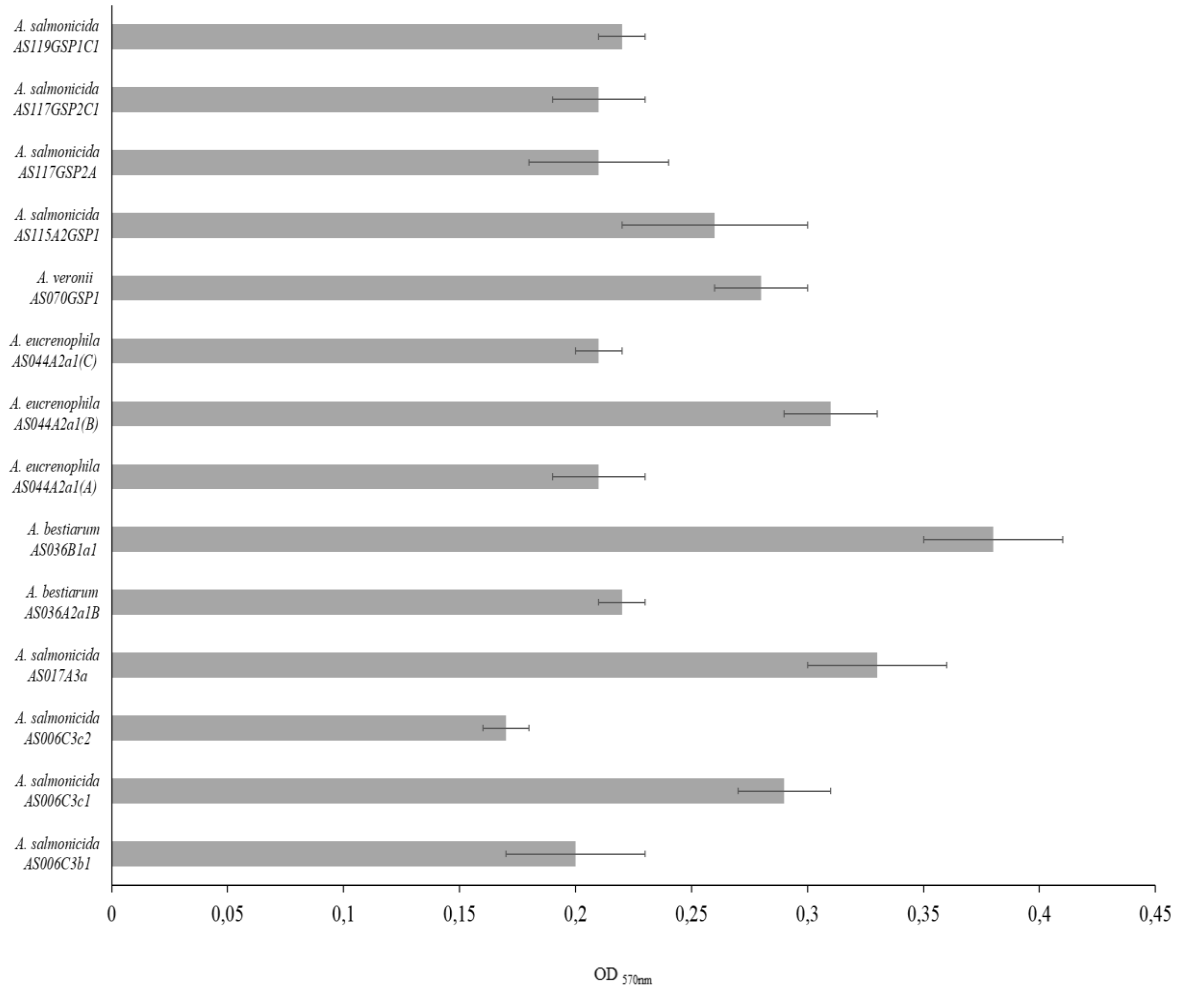

Figure 5.2 Values of $OD_{570\ nm}$ as a measure of monolayer bacterial adhesion on PS for 2 h. The mean ± SD of three independent experiments are illustrated.

Of all strains, 57% (8/14) were classified as moderately adherent to PS. *A. bestiarum* AS036B1a1 and *A. salmonicida* AS006C3c2 had the highest and lowest adhesion abilities, respectively (Table 5.3).

Table 5.3 Adhesion and biofilm formation ability of *Aeromonas* strains to PS according to the classification proposed by Stepanović et al. (2000)

Source wild animal	Species	Adhesion	Biofilm		
			24 h	48 h	72 h
Cervus elaphus (Red deer)	*A. salmonicida* AS006C3b1	+	+	+++	+++
	A. salmonicida AS006C3c1	++	+	+++	+++
	A. salmonicida AS006C3c2	+	+	+	+
	A. salmonicida AS115A2GSP1	++	+	++	+++
	A. salmonicida AS117GSP2A	+	+	++	++
	A. salmonicida AS117GSP2C1	+	+	+	++
	A. salmonicida AS119GSP1C1	++	+	++	++
Circaetus gallicus (Short-toed snake eagle)	*A. veronii* AS070GSP1	++	++	+	++
Colubridae (Snake)	*A. eucrenophila* AS044A2a1(A)	+	+	++	++
	A. eucrenophila AS044A2a1(B)	++	+	+	++
	A. eucrenophila AS044A2a1(C)	+	+	+	++
Sciurus vulgaris (Red squirrel)	*A. bestiarum* AS036A2a1B	++	+	++	++
	A. bestiarum AS036B1a1	++	+	++	++
Strix aluco (Tawny owl)	*A. salmonicida* AS017A3a	++	+	+	+

(0) - non-adherent/non-biofilm producer; (+) - weakly adherent/weak biofilm producer; (++) - moderately adherent/moderate biofilm producer; (+++) - strongly adherent/strong biofilm producer

5.3.4 Biofilm formation

The total biomass of biofilms was quantified by staining with CV. Figure 5.3 shows biomass of biofilms with different ages (24, 48 and 72 h). The results show that all strains were able to form biofilms, however, they had statistically distinct productivities ($P < 0.05$). *A. salmonicida* AS006C3c1 produced the highest biomass amount for 72 h, *A. salmonicida* AS006C3b1 for 48 h and *A. veronii* AS070GSP1 for 24 h sampling times. *A. eucrenophila* AS044A2a1(B), *A. salmonicida* AS017A3a and *A. salmonicida* AS006C3c2 produced the lowest biomass amount for 24, 48 and 72 h sampling times, respectively.

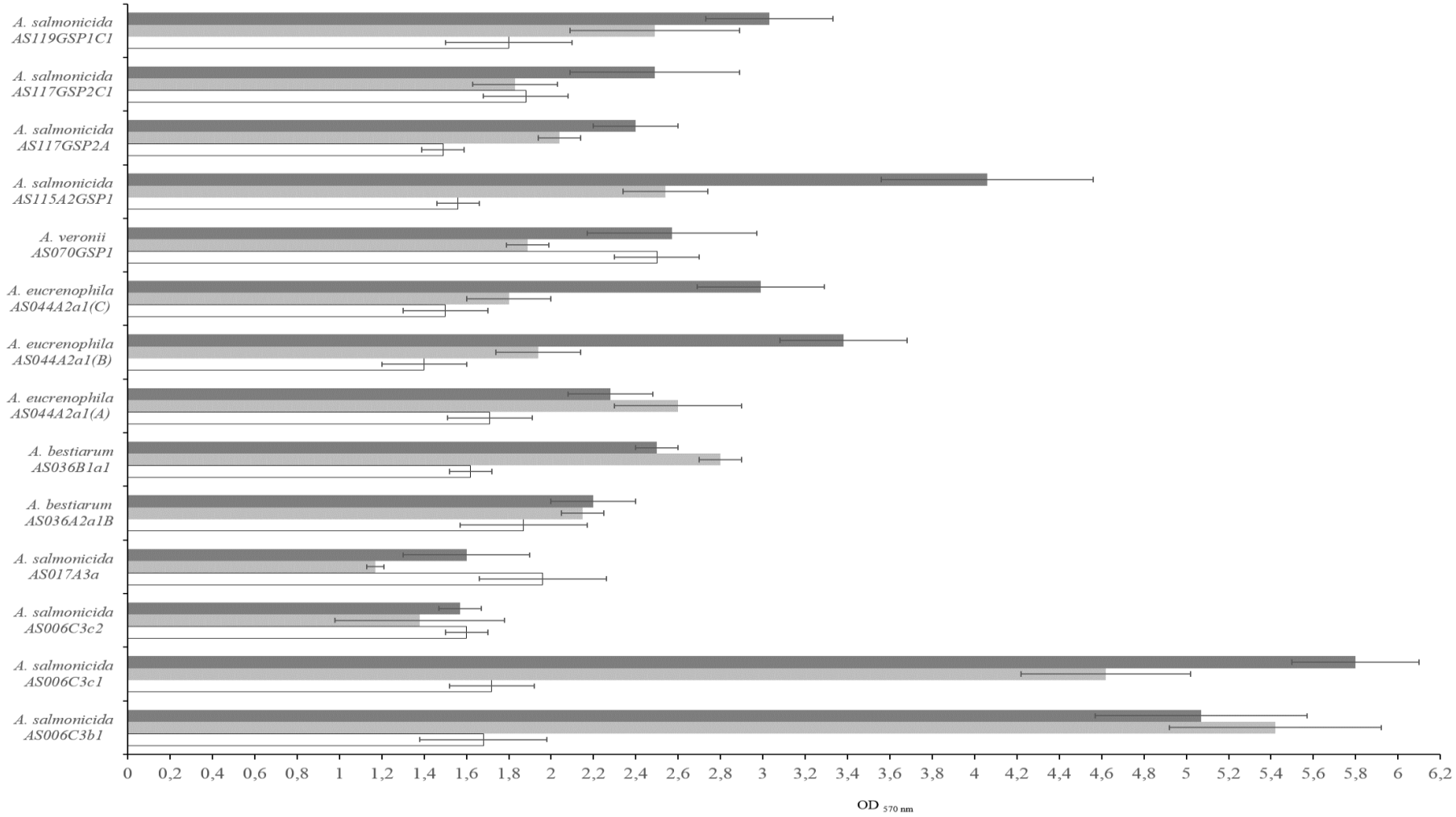

Figure 5.3 Values of $OD_{570\,nm}$ as a measure of biomass of 24 h (□), 48 h (▒) and 72 h (■) hours old biofilms. The mean ± SD of three independent experiments are illustrated.

The diverse strains were classified in terms of biofilm productivity as weak, moderate or strong biofilm producers (Table 5.3). *A. salmonicida* AS006C3b1 and *A. salmonicida* AS006C3c1 showed a strong ability of biofilm formation at 48 and 72 h sampling times and *A. salmonicida* AS115A2GSP1 showed a strong ability of biofilm formation at 72 h sampling time.

A. salmonicida AS006C3c2 and *A. salmonicida* AS017A3a showed a weak ability of biofilm formation for all sampling times. *A. veronii* AS070GSP1 showed variability in the ability of biofilm formation, showing a moderate ability at 24 h, a weak ability at 48 h and a moderate ability again at 72 h. *A. eucrenophila* AS044A2a1(A) showed a weak ability of biofilm formation at 24 h and moderate ability at 48 and 72 h.

5.3.5 Ciprofloxacin (CIP) on biofilm removal

The ability of CIP to control 24 h-old biofilms was analyzed in terms of biomass removal (Figure 5.4). Moderate removal (> 25%) was found for nine strains (*A. salmonicida* AS006C3b2, *A. salmonicida* AS006C3c1, *A. salmonicida* AS006C3c2, *A. salmonicida* AS017A3a, *A. bestiarum* AS036A2a1B, *A. bestiarum* AS036B1a1, *A. eucrenophila* AS044A2a1(A), *A. eucrenophila* AS044A2a1(B) and *A. veronii* AS070GSP1). The most significant removal, with statistical significance compared to other strains ($P < 0.05$) was found for biofilms of *A. salmonicida* AS006C3c2 (52%), *A. bestiarum* AS036B1a1 (56%) and *A. eucrenophila* AS044A2a1(B) (52%). Ciprofloxacin had low effects (removal < 25%) on the removal of biofilms formed by the other strains. In fact, no biomass removal was observed for biofilms formed by *A. eucrenophila* AS044A2a1(C), *A. salmonicida* AS117GSP2A, *A. salmonicida* AS117GSP2C1 and *A. salmonicida* AS119GSP1C1.

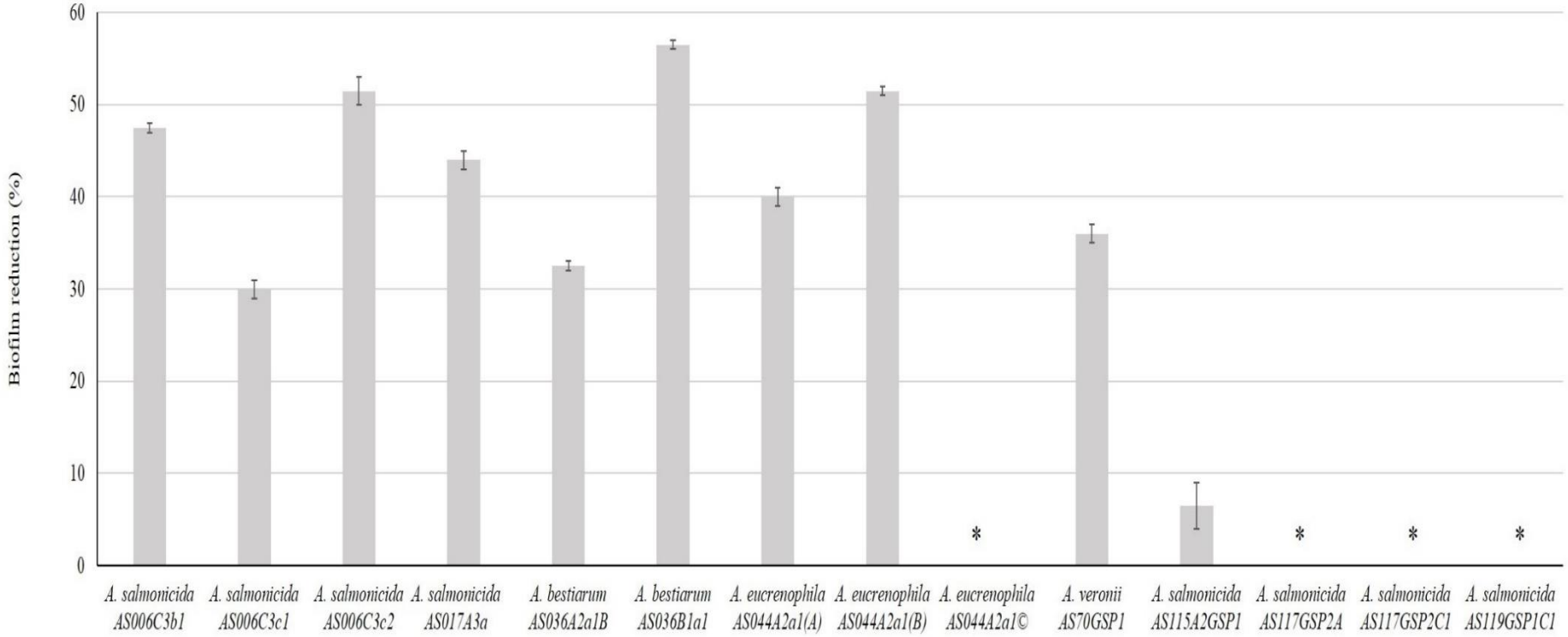

Figure 5.4 Percentage of biofilm reduction for the *Aeromonas* strains exposed to 10× MIC of ciprofloxacin for 24 h. * - Means no biofilm removal. Mean values ± SD for at least three replicates are illustrated.

5.4 DISCUSSION

Wild animals can acquire resistant bacteria acting as potential reservoirs, vectors and/or bioindicators of antimicrobial resistant pathogens (Schaefer *et al.*, 2009, Carroll *et al.*, 2015). Numerous studies have demonstrated that approximately 60% of the emerging infectious diseases in humans are zoonotic (Ahmed *et al.*, 2007, Taylor *et al.*, 2011, Cantasand Suer, 2014). Moreover, about 70% of zoonotic infections are considered from wildlife origin (Kruse *et al.*, 2004, Haagmans *et al.*, 2009, Cutler *et al.*, 2010, Wu *et al.*, 2016).

Aeromonas spp. can be commonly found in a variety of aquatic environments (Pianetti *et al.*, 2005, Jandaand Abbott, 2010). However, increasing data suggest that animals are an ever-present reservoir for the introduction and exchange of *Aeromonas* species in the environment (Jandaand Abbott, 2010). Mesophilic *A. veronii*, *A. eucrenophila* and *A. bestiarum* species cause a similar variety of diseases in fish (Jandaand Abbott, 2010). Ceylan *et al.* (2009) reported that *Aeromonas* species were identified in the gastrointestinal contents of sheep, cattle, and horses at frequencies ranging from 5 to 10%. *Aeromonas* spp. are also recognized as etiologic agent responsible for a variety of infection in both immunocompetent and immunocompromised persons (Jandaand Abbott, 2010).

In the present study, 14 different *Aeromonas* strains were isolated from five distinct wild animal species. These strains were already found to be resistant to *β*-lactam antibiotics, therefore representing a potential risk to public health.

The treatment of *Aeromonas* infections can be done with different classes of antibiotics, particularly *β*-lactam, chloramphenicol and tetracycline antibiotics (Ko *et al.*, 2003). However, an increase in resistance to those antibiotics among disease-causing *Aeromonas* species was reported (Igbinosa *et al.*, 2015). In the present study, the susceptibility of the *Aeromonas* strains to ciprofloxacin was assessed. This antibiotic was selected due to its current use for the treatment of human and animal bacterial infections (Riddle *et al.*, 2000, Farnell *et al.*, 2005)

Quinolones are broad spectrum synthetic antibiotics with recognized efficacy against *Aeromonas* spp. (Alcaide *et al.*, 2010). However, many studies have indicated that members of the genus *Aeromonas* can harbor genes encoding *β*-lactam and plasmids conferring quinolone resistance, with the potential to spread via horizontal gene transfer (Cattoir *et al.*, 2008, Moura *et al.*, 2012). Resistance to ciprofloxacin can be a potential public health threat since quinolones are used for the treatment of *Aeromonas* infection in human and veterinary medicine and in agriculture (Alcaide *et al.*, 2010). Quinolone-resistant strains of *Aeromonas*

recovered from humans, freshwater and eels were previously reported (Arias *et al.*, 2010). In particular, strains of *A. caviae* and *A. veronii* isolated from stools of patients with gastrointestinal symptoms were resistant to nalidixic acid (Arias *et al.*, 2010). The MIC of ciprofloxacin varied from 6 to 60 µg/ mL. According to the MIC breakpoint of ≥ 4 µg/ mL for ciprofloxacin-resistant *Aeromonas* species (CLSI, 2014), all strains tested in this study are considered resistant to ciprofloxacin. These results together with the resistance patterns of previous antibiotic susceptibility tests (Dias *et al.*, 2014) indicate that all strains under study are resistant to more than two antibiotics. Previous studies (Alcaide *et al.*, 2010, Arias *et al.*, 2010) reported quinolone-resistant strains of *Aeromonas* recovered from humans, freshwater and eels. Of particular concern was the detection of *A. caviae* and *A. veronii* strains, isolated from stools of patients with gastrointestinal symptoms, and resistant to nalidixic acid (Arias *et al.*, 2010). To our knowledge this is the first report showing multiresistant *Aeromonas* isolated from wild animals.

Aravena-Román et al. (2012) described that 144 clinical strains and 44 environmental strains of *Aeromonas* spp. were all inhibited by ciprofloxacin with MIC of 1 µg/ mL, for all strains. More recently, Dobiasova et al. (2010) reported MIC of ciprofloxacin from 0.25 to > 180 µg/ mL against *Aeromonas* strains. Differences in antibiotic susceptibility were also reported for strains of the same species. The wild animals can be infected or colonized by multiresistant strains, although they have only a rare contact with antibiotics. The major mechanism of bacteria transmission of bacteria of veterinary animal or human origin to wild animals can be through water contact and acquisition via food (Cole *et al.*, 2005, Kozak *et al.*, 2009). Wild animal in general could therefore serve as reservoirs of resistant bacteria and genetic determinants of antimicrobial resistance (Dolejska *et al.*, 2007).

Biofilm formation results in increased resistance to antibiotics and in persistent infections affecting both animal and human health (Behlauand Gilmore, 2008, Castro *et al.*, 2014). Bacterial adhesion to host tissues is an essential initial step in the infection process. Although its role in infection appears to be linked to biofilm formation (Jandaand Abbott, 2010), knowledge on the factors involved in biofilm formation by environmental *Aeromonas* spp. is limited. Bacteria isolated from different niches exhibit different abilities to adhere on the substratum and form biofilms, that depends not only on the surface characteristics and the environment surrounding the bacterial cells (nutrients, ionic strength, pH and temperature) but also on their phenotype and genotype (Thomas *et al.*, 2002). Several studies indicated that *Aeromonas* spp. are able to adhere to different surfaces such as stainless steel, copper, polybutylene and rubber (Kirov *et al.*, 2004, Kregiel, 2013).

The *Aeromonas* strains were characterized based on their surface properties and their abilities for adhesion and biofilm formation. It is widely accepted that bacterial surface hydrophobicity is a key factor in the adhesion process and further biofilm development (Deere *et al.*, 1997, Kregiel, 2013). In this study, the tested *Aeromonas* strains adhered on PS, however, they exhibited different adhesive strengths regardless of their animal source or bacterial species.

In order to evaluate whether the initial bacteria-PS binding event was determinant for subsequent biofilm development, the ability of the isolated strains to form biofilms was evaluated. PS microtiter plates were used in this study as standard bioreactor system for adhesion and biofilm formation. This type of device was used in similar studies with bacteria from different environments providing reliable comparative data (Simões *et al.*, 2010, Monte *et al.*, 2014). No clear relationship was found between the amount of biofilm formed and initial monolayer adhesion extent. For instance, strains *A. salmonicida* AS006C3b1, *A. eucrenophila* AS044A2a1(A), *A. eucrenophila* AS044A2a1(C), *A. salmonicida* AS117GSP2A and *A. salmonicida* AS117GSP2C1, had weak monolayer adhesion ability; however, these strains were classified as moderate biofilm formers after 48 h of growth in microtiter plates.

These results suggest that biofilm formation is more likely to be influenced by cell-cell aggregation and interactions than by the initial adhesion of the strains to the substratum (Alves *et al.*, 1999). The data from the present study demonstrates that each strain had a different ability to form biofilms, even under the same experimental conditions. This result proposes that single strains cannot represent the behavior of a species. A similar conclusion was obtained by Chenia and Duma (2016) when studying the biofilm formation ability of *Aeromonas* strains isolated from diverse freshwater fish species and from sea water.

In a recent study OrmancandYucel (2017) reported biofilm formation by diverse *Aeromonas* species (food and environmental isolates) during 24 h and found $OD_{570\,nm}$ ranging from 0.090 and 1.255. It is important to refer that different growth media were used for biofilm formation: MHB – selected as standard medium for antibiotic testing (CLSI, 2014) and Tryptic Soy Broth-TSB and Brain-Heart Infusion-BHI). In fact, Ormanci and Yucel (2017) proposed that that different growth media, due to their diverse ingredients, had distinct effects on biofilm formation, with less-rich growth medium (TSB) favouring biofilm formation. Dewanti and Wong (1995) also found that *Escherichia coli* developed biofilm faster and with a higher cell density when grown in low-nutrient media. Hood and Zottola (1997) demonstrated that *Salmonella typhimurium* produce more biofilm in nutrient-limited media, than *Listeria monocytogenes*, which produce more biofilm in nutrient-rich medium.

In fact, bacterial adhesion to surfaces and biofilm formation is dependent on the specific strain and is not a general characteristic of a bacterial species or serotype (Santiago *et al.*, 1999, Elhariry, 2011). Biofilm formation ability was strongly dependent on the time, i.e. longer biofilm formation periods increased biomass productivity, except for *A. salmonicida* $_{AS017A3a}$ and *A. salmonicida* $_{AS006C3c1}$ strains. After 24 hours of incubation the majority of strains (86%) had weak ability to form biofilm. Only strain *A. veronii* $_{AS070GSP1}$ had moderate biofilm formation ability. Results of Igbinosa et al. (2013) showed that 53% of *Aeromonas* strains isolated from two rivers were weak biofilm producers, 29% were strong producers while 16% demonstrated moderate ability to form biofilms. Odeyemi et al. (2012) reported that *A. hydrophila* isolated from estuaries were able to form biofilms, even if most of the strains were weak biofilm producers. In the present study, using four different *Aeromonas* species, a similar behavior was observed. The majority of strains (except *A. veronii* $_{AS070GSP1}$, classified as moderate biofilm producer) had weak biofilm producing ability at 24 h sampling time. Increasing the biofilm formation period also increased biomass production, for most of the cases, with the exception of *A. salmonicida* $_{AS006C3c2}$, *A. salmonicida* $_{AS017A3a}$ and *A. veronii* $_{AS070GSP1}$. For instance, *A. salmonicida* $_{AS006C3b1}$ and *A. salmonicida* $_{AS006C3c1}$ were classified as weak biofilm producer at 24 h and strong biofilm producers at 48 and 72 h. The presence of *Aeromonas* spp. with biofilm-producing potential is of great concern to public health because these organisms can harbour other pathogenic microorganisms (Jandaand Abbott, 2010). Studies of Kirov et al. (2004) comparing *in vitro* biofilm assays and biofilm formation on human tissues, reported that the ability of *Aeromonas* strains to form biofilms further reveals their pathogen status.

Due to the recognized higher resistance of sessile communities when compared with their planktonic counterparts (Simões *et al.*, 2010), biofilms were exposed to ciprofloxacin with 10 times above the MIC. This treatment caused biofilm mass reductions from 0 to 58%. Lewis (2001) proposed that the majority of biofilm cells were killed within a ciprofloxacin concentration ≤ 5 µg/ mL. In another investigation, Walters et al. (2003) studied the effect of ciprofloxacin (1.0 µg/ mL) against *Pseudomonas aeruginosa* biofilms and observed that the antibiotic penetrated biofilms but failed to kill the bacteria. Later, Lewis (2007) observed that fluoroquinolones are effective in preventing biofilm formation, but once the biofilm community is mature the antimicrobial agent is restricted in its ability to diffuse through the biopellicle and reach the target site. This is the probable explanation for the reduced effects of ciprofloxacin in biofilm removal found in this study. In fact, biofilms are heterogeneous and complex structures of microorganisms presenting sophisticated singular and collective

behaviours which allows the colonizing microorganisms to survive in hostile environments (Simões, 2011).

5.5 CONCLUSION

In conclusion, this work demonstrates that *Aeromonas* strains isolated from wild animals have the ability to adhere on PS and form biofilms. Initial adhesion and biofilm formation on abiotic surfaces are important virulence mechanisms of bacteria to facilitate survival within host cells and in adverse environmental conditions. These characteristics are strain-dependent and do not represent a species-specific phenotype or correspond to the source of bacterial strains. Moreover, there is an extensive range of variation in adhesion and biofilm formation among the *Aeromonas* strains. The 14 strains were resistant to ciprofloxacin and multiresistant. Moreover, the use of ciprofloxacin with 10 times above the MIC was not effective in biofilm removal. Therefore, the overall results propose that the strains tested are of potential public health concern. In fact, most *Aeromonas* strains are consistently considered opportunistic pathogens and the ability to form biofilms will increase their antimicrobial resistance, and their potential to cause persistent infections.

CHAPTER 6. BIOFILM FORMATION AND ANTIBIOTIC SUSCEPTIBILITY OF MULTIRESISTANT BACTERIA ISOLATED FROM WILD ANIMALS

DIAS, C., BORGES, A., MARTINEZ-MÚRCIA, A., SAAVEDRA, M.J., SIMÕES, M. Biofilm formation and antibiotic susceptibility of multidrug-resistant bacteria isolated from wild animals. *(Submitted)*

ABSTRACT

The emergence and spread of multiresistant microorganisms in wildlife are growing concerns with serious implications for both animal and human health. The One Health initiative recognizes that the health of people is connected to the health of animals and the environment. However, information on the resistance and resilience of microorganisms from wild animals is scarce. The purpose of this work was to determine the antibiotic susceptibility of four strains (*Acinetobacter* spp. AS027A3a, *K. pneumonia*e AS0027A2, *P. fluorescens* AS008A1 and *S. putrefaciens* AS006C2) isolated from wild animals and their ability to adhere and form biofilms on polystyrene. The effect of two antibiotic (imipenem - IPM and ciprofloxacin - CIP) on biofilm removal was also tested. The susceptibility profile demonstrated that they are multiresistant to antibiotics. Based on the *bla*-encoding genes, three *β*-lactamases were detected in *K. pneumoniae* AS0027A2 (TEM, SHV and OXA-aer) and one in *P. fluorescens* AS008A1 (OXA-aer). No relationship was found between monolayer surface adhesion and biofilm formation. *P. fluorescens* AS008A1 was the strain with the highest adhesion ability while *K. pneumoniae* AS0027A2 was the strain producing more biofilm. In general, the antibiotics at their minimum inhibitory concentrations (MIC) and at 10× MIC were ineffective in total biofilm removal. The highest biomass reductions were found with IPM (54% at 10× MIC) against *K. pneumoniae* AS0027A2 biofilms and with CIP (40% at 10× MIC) against *P. fluorescens* AS008A1 biofilms. These results suggest that wildlife comprise important host reservoirs and vectors for the spread of multidrug-resistant bacteria and genetic determinant of resistance.

Keywords: biofilm, *β*-lactamases, multidrug-resistant, wild animal.

6.1 INTRODUCTION

Tons of antibiotic residues are discarded into natural resources every year, making the environment a reservoir of resistance genes (Daviesand Davies, 2010). Moreover, antibiotic resistance is a natural phenomenon with microorganisms from the environment and human pathogens sharing the same resistome (Dantas *et al.*, 2008, Forsberg *et al.*, 2012).

In the last years it has been observed a drastic increase both in the proportion and absolute number of pathogenic bacterial strains resistant to a large number of antibiotics (Finley *et al.*, 2013). These include *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and other Gram-negative species pathogenic to humans (Finley *et al.*, 2013). In Gram-negative bacteria, one of the most important mechanism of antibiotic resistance is the production of *β*-lactamases (Hawkeyand Jones, 2009). These enzymes are usually acquired by horizontal gene transmission and confer resistance to *β*-lactam, the most commonly used class of antibiotics for the treatment of human and animal infections (Henriques *et al.*, 2006).

Another major factor contributing to antibiotic resistance is the ability of the microorganisms to form sessile communities on biotic or abiotic surfaces, commonly known as biofilms (Hoiby *et al.*, 2010). Biofilms can be defined as a dynamic biological system of microbial cells that are irreversibly associated with a surface, and embedded in an organic polymeric matrix of microbial origin (Jacques *et al.*, 2010). It is estimated that more than 65% of microbial infections are caused by microorganisms in biofilms (Cookand Dunny, 2014). It is known that bacterial cells during their transition from planktonic to biofilm structure undergo extensive changes. These changes are reflected in the phenotypic characteristics of the biofilms cells (O'Toole *et al.*, 2000). Resistance mechanisms described for planktonic cells such as antibiotic modifying enzymes, target modification, and efflux pumps, do not explain the high resistance of biofilm cells (Vanegas *et al.*, 2009). The occurrence of antibiotic resistance in biofilms is not fully known, but several works have used a variety of model systems to understand how and why biofilms are less susceptible to antibiotics (Mahand O'Toole, 2001). The prosed mechanisms include physical or chemical diffusion barrier to antibiotic penetration into the biofilms and low growth rate of the biofilms cells owing to nutrient limitations, the presence of persister cells (de la Fuente-Nunez *et al.*, 2013). The resistance of biofilms varies from one microorganism to another and in most cases is a combination of several

mechanisms (Andersonand O'toole, 2008, Dufour *et al.*, 2012). The World Health Organization (WHO) recently recognised antibiotic resistance as a serious global problem not only in terms of human health but also for the animals (both domestic and wildlife) and environmental health (Gibbs, 2014). Indeed, bacteria can move easily between different ecosystems, from animals and humans to water and soil. Besides, the exchanges of resistance genes among bacterial strains from different environments can also occur (da Costa *et al.*, 2013). Several studies have demonstrated that antibiotic-resistant strains are present in a wide variety of ecological niches, including wild bird species that habit in remote ecosystems (Smith *et al.*, 2014). However, the knowledge on the propensity of these strains to establish sessile communities and on the consequent advantages to survive in adverse conditions is scarce (Daviesand Davies, 2010).

This study characterizes phenotypically and genotypically the antibiotic resistance pattern of four bacterial strains (*Shewanella putrefaciens* AS006C2, *Pseudomonas fluorescens* AS008A1, *Klebsiella pneumoniae* AS027A2 and *Acinetobacter* spp. AS027A3a) isolated from fecal samples of different species of wild animals. Their ability to form biofilms was assessed. The effects of the antibiotics ciprofloxacin (CIP) and imipenem (IPM) was assessed in biofilm removal.

6.2 MATERIALS AND METHODS

6.2.1 Sample collection and bacterial isolation

Bacterial strains were collected aseptically, directly from the animal rectum or from freshly voided faecal material, of different species of wild animals (birds, reptiles and mammals) of the north of Portugal according to the procedure described in sub-section 3.1. MacConkey agar (Merck, Germany) was used for bacterial isolation and the plates incubated at 37 ºC for 24 h.

6.2.2 Antibiotic susceptibility testing

The antimicrobial sensitivity profile of recovered bacteria was determined using a disk diffusion assay on Muller-Hinton agar (MH, Oxoid, UK), according to the standards and interpretive criteria described by the Clinical and Laboratory Standards Institute guidelines (Sub-section 3.2.2).

6.2.3 Identification of bacterial resistant strains

Identification was obtained by 16S rRNA gene sequence analysis. For that, general bacterial 5`GGT TAC CTT GTT ACG ACT T 3`reverse primer and 5`AGA GTT TGA TCA TGG CTC AG3` forward primer were used to amplify nearly full-length 16S rRNA gene as described previously (Martínez-Murcia *et al.*, 1999, Soler *et al.*, 2004). Sequencing analysis were performed using the BigDye Terminator V3.1 cycle sequencing kit ant the ABI Prism® 3100 Avant Genetic Analyzer (Applied Biosystems, USA) (Sub-section 3.2.2).

6.2.4 Minimal inhibitory concentration (MIC) determination

Standardized planktonic MIC´s of the CIP (Sigma-Aldrich Co., Portugal) and IPM (Cayman Chemical, USA) antibiotics were assessed by the microdilution method outlined by CLSI (2014). Overnight cell cultures (16-18 h incubation) were adjusted to a cell density (600 nm) of 1×10^6 CFU/ mL and added to sterile 96-well polystyrene microtiter plates (Orange Scientific, USA) containing different concentration of CIP (6, 8, 10, 14, 16, 24, 32, 36, 40, 44, 52, 56 and 60 µg/ mL) and IPM (2, 4, 6, 8, 10, 12 µg/ mL) in a final volume of 200 µL. A bacterial suspension without antibiotic was used as negative control. Microtiter plates were then incubated at 30 ºC for 24 h. MIC matched to the concentration in which the final optical density (OD) at 629 nm was equal or lower than the initial OD (Sub-section 3.3.2).

6.2.5 Screening of resistance genetic determinants: *β*-lactamases

Detection of genetic determinants was done for TEM-type, SHV-type, CTX-M-type, MOX and FOX variants of extended-spectrum-*β*-lactamases (ESBLs) and for the CphA, IMP-type, VIM-type and OXA-type carbapenemases. The mixture of PCR was performed in a final volume of 50 µL containing 5 µL of Biotools PCR buffer (10×), 200 µM of each nucleotide, 10 pmol of each primer (Table 3.5, Sub-section 3.4), 1 µL (1U) of Taq DNA polymerase (Biotools) and 1 µL of template genomic DNA. The amplification conditions were: initial denaturation for 5 min at 95 ºC, 35 amplification cycles composed of a denaturation step for 15 seconds at 94 ºC, an annealing step at 55 ºC for 30 seconds, except for bla_{FOX}, in which 60 ºC were used, and an extension

period at 72 ºC for 45 seconds. The reaction was completed with an extension step at 72 ºC for 3 min.

6.2.6 Bacterial surface hydrophobicity

Surface hydrophobicity was evaluated after contact angle measurement, according to the procedure described by Simões *et al.* (2010). Lawns of each strains were prepared as described by Busscher *et al.* (1984) and their contact angles contact angles were determined by the sessile drop contact angle measurements using a model OCA 15Plus (DATAPHYSICS, Germany), with three pure liquids (water, formamide, and α-bromonaphthalene). The surface tension components, of the three liquids, were obtained from the literature. Afterwards, the hydrophobicity and surface tension parameters (Υ^{LW}, Υ^{AB}, Υ^{+}, Υ^{-}) of the strains were evaluated according to the method of van Oss *et al.* (1989) (Sub-section 3.7).

6.2.7 Initial adhesion ability

In vitro initial adhesion, was determined according to the method of Simões *et al.* (2007) (Sub-section 3.9.2). Sterile microtiter plates (12-well) with 2 mL of cell suspension (1×10^8 cells/ mL in MH) and coupons of PS were incubated at 30 ºC for 2 h in an orbital shaker at 150 rpm. In order to prepare the PS coupons, they were immersed in a solution (5% v/v) of commercial detergent (Sonasol Pril, Henkel Ibérica S.A) and ultrapure water with gentle shaking (30 min). All assays were performed in triplicate and with three repeats. After 2 h incubation the coupons were removed and washed in 2 mL of sterile saline solution (0.85% w/v) and the biomass was quantified by crystal violet (CV) staining (Simões *et al.*, 2010).

6.2.8 Biofilm formation

Biofilm formation was performed in 96-well polystyrene microplates following the method of Stepanović *et al.* (2000) (Sub-section 3.10). Briefly, overnight cultures were adjusted to an initial OD (620 nm) of 0.04 ± 0.02 (1×10^8 cells/ mL) in MH and 200 µL aliquots were added to the microplate (least sixteen wells). The microtiter plate were incubated for 24, 48 and 72 h at 30 ºC and agitated at 150 rpm. The medium (MH)

was carefully and aseptically replaced by fresh one daily. Negative control wells containing MH broth without strains.

6.2.9 Biofilm control

To determine whether the antibiotic CIP and IPM had capability to remove 24 and 48 h-aged biofilms 5× MIC and 10× MIC were used according to Simões *et al.* (2010). After 24 h of exposure the biofilms were analyzed in terms of biomass by CV staining (Simões *et al.*, 2010) and the results presented as percentages of biofilm mass removal, according to described in sub-section 3.11. All tests were performed in triplicate with three independent experiments.

6.2.10 Mass quantification of adhered cells and biofilms

The mass of adhered cells and biofilm cells was quantified by CV staining according to Simões *et al.* (2010) (Sub-section 3.12) . The OD was measured at 570 nm using a Microplate Reader (BIO-TEK, Model Synergy HT). Biofilm mass removal percentage (% BR) was given by the equation 8.

Where the ODc are the absorbance values of the biofilms non-exposed to the antibiotics (CIP and IPM) and ODw are the absorbance values for biofilms exposed to antibiotics (CIP and IPM).

2.6.11 Adherent/biofilm bacteria classification

Based on upon the cutoff of the ODc quantification, the bacteria was classified according to the scheme previously stated in sub-section 3.13. This classification was based upon the cut-off of the optical density (ODc) value defined as three standard deviation values above the mean OD of the negative control (Stepanović *et al.*, 2000).

2.6.12 Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using the statistical program SPSS version 22.0 (Statistical Package for the Social Sciences) (sub-section 3.16)

6.3 RESULTS

6.3.1 Bacterial strains and antibiotic susceptibility profile

The selected strains belong to different genus (*Shewanella*, *Pseudomonas*, *Klebsiella* and *Acinetobacter*) and were obtained from fecal samples of wild animals of the north of Portugal (Table 6.1). *S. putrefaciens* AS006C2 was obtained from a sample of dee (*Cervus elaphus*), *P. fluorescens* AS008A1 from a fox (*Vulpes vulpes*) and the strains *K. pneumoniae* AS0027A2 and *Acinetobacter* spp. AS027A3a were isolated from the same sample of a greater rhea (*Rhea americana*).

They were selected for further 16S rRNA identification due to their resistance profile. 16S rRNA sequences of these strains were compared to the GenBank nucleotide data library using the BLAST software of the National Center of Biotechnology Information website (NCBI) (http://www.ncbi.nlm.nih.gov/) in order to observe their unknown phylogenetic affiliation. The phylogenetic analysis revealed that three strains are identified as *P. fluorescens* AS008A1, *K. pneumonia* AS027A2 and *S. putrefaciens* AS006C2. The strain *Acinetobacter* spp. AS027A3a was only identified at the genus level. According to the analysis of the phylogenetic tree generated (Figure 6.1), there are evidences that this strain can represent a new species.

The susceptibility profile against 27 antibiotic belonging to different classes were performed. These antibiotics included: penicillins (aminopenicillins and carboxypenicillins), cephalosporins (1st, 3rd and 4th generation), monobactams, carbapenems, tetracyclines, macrolides, quinolones, aminoglycosides, chloramphenicols and fosfomycins. Dual combinations of two antibiotics (trimethoprim/sulfamethoxazole) and antibiotics with β-lactamases inhibitors (ticarcillin/clavulanic acid and piperacillin/tazobactam) were also tested. Table 6.1 shows the resistance phenotype of the four strains. All the strains were multiresistant, i.e. resistant to at least two antibiotic belonging to different chemical classes. It was possible to observe that the two strains (*Acinetobacter* spp. AS027A3a and *K. pneumoniae* AS027A2) isolated from the same sample showed identical resistance profile. Among the strains isolated *S. putrefaciens* AS006C2 was the most susceptible to the antibiotics tested. All strains were resistant to amoxicillin, amoxicillin/clavulanic acid, cephalothin, fosfomycin and erythromycin. Of particular concern was the resistance of all the strains to carbapenem IPM, an intravenous broad-spectrum antibiotic of the β-lactams group that is exclusively of hospital use (Papp-Wallace *et al.*, 2011).

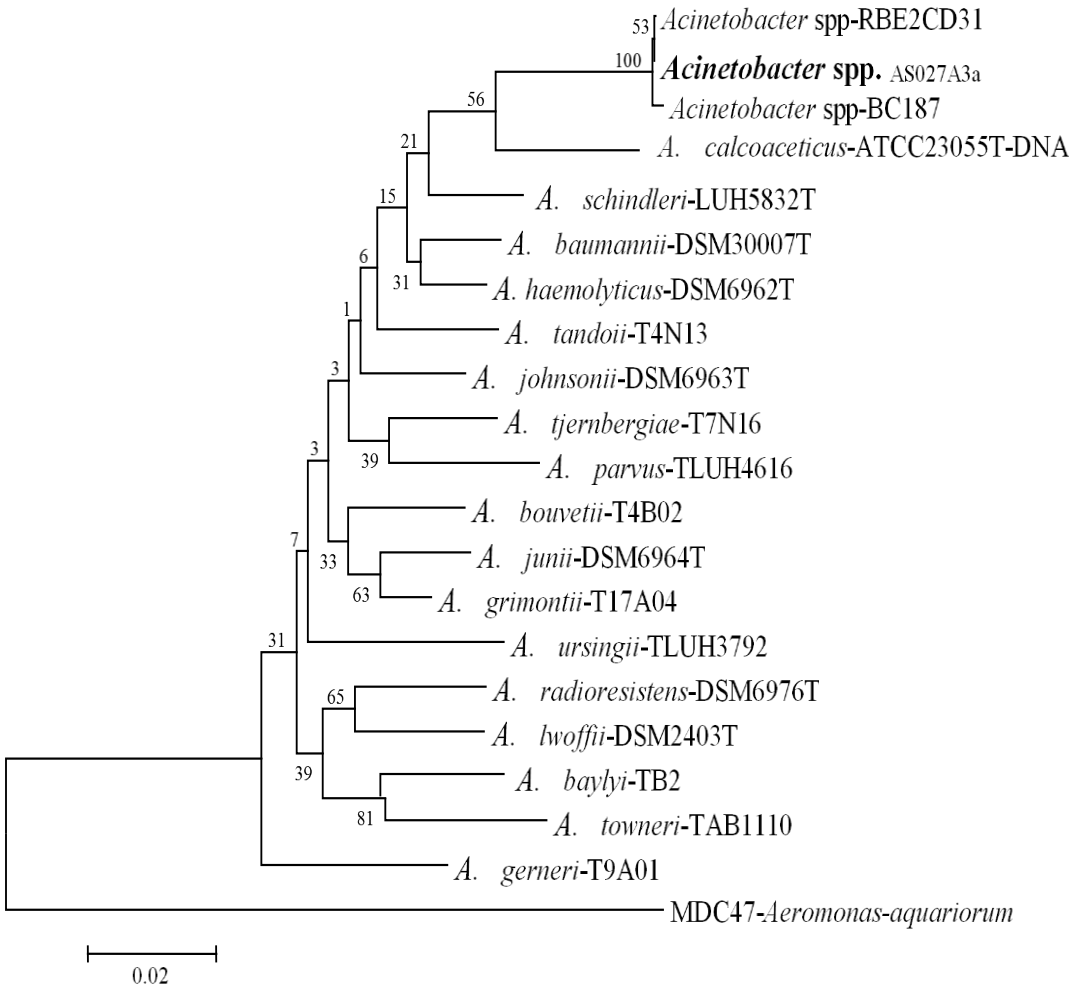

Figure 6.1 Unrooted phylogenetic tree based on 16S rDNA gene sequences of *Acinetobacter* spp. AS027A3a strain isolated in this study and the bacterial strains of the Nacional Center for Biotechnology Information (NCBI) databases.

6.3.2 Genetic bases of antibiotic resistance

The presence of sequence β-lactamases genes (*bla*$_{\text{CphA}}$; *bla*$_{\text{OXA-aer}}$; *bla*$_{\text{OXA-B}}$; *bla*$_{\text{OXA-C}}$; *bla*$_{\text{MOX}}$; *bla*$_{\text{TEM}}$; *bla*$_{\text{FOX}}$; *bla*$_{\text{SHV}}$; *bla*$_{\text{SHV}}$; *bla*$_{\text{CTX-M}}$; *bla*$_{\text{IMP}}$ and *bla*$_{\text{VIM}}$) in the selected strains was analyzed by PCR. The genotype results for the studied strains are summarized in Table 6.1. The *bla*$_{\text{OXA-aer}}$ was detected in *P. fluorescens* AS008A1 and *K. pneumoniae* AS027A2, strains isolated from fecal samples of a fox and an emu, respectively. Three β-lactamases genes (*bla*$_{\text{OXA-aer}}$; *bla*$_{\text{TEM}}$; *bla*$_{\text{SHV}}$) were detected in the same strain, *K. pneumoniae* AS027A2. *Acinetobacter* spp. AS027A3a and *S. putrefaciens* AS006C2 were resistant to IPM but apparently did not display any genetic determinants of IPM

resistance used in this work. No PCR specific *bla*$_{CphA}$, *bla*$_{OXA-B}$, *bla*$_{OXA-C}$, *bla*$_{MOX}$, *bla*$_{FOX}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ encoding sequences were detected.

Table 6.1 Characterization of the bacterial strains isolated as concerns resistance phenotypes and *bla* genotypes

Animal species names	Strains identification (*16s rRNA* gene sequencing)	Resistance phenotype	*β*-lactamase genes content
R. americana (Greater rhea)	*Acinetobacter* spp. AS027A3a	AML, AMC, TIM, ATM, IPM, KF, FOS, FOX, CFP, CIP, S, E, C, SxT, TE	-
	K. pneumoniae AS027A2	AML, AMC, TIM, ATM, IPM, KF, FOS, FOX, CFP, CIP, S, E, C, SxT, TE	*bla*$_{OXA-aer}$; *bla*$_{TEM}$; *bla*$_{SHV}$
V. vulpes (Fox)	*P. fluorescens* AS008A1	AML, AMC, TIC, TIM, ATM, IPM, KF, FOS, FOX, CTX, E, C, SxT, TE	*bla*$_{OXA-aer}$
C. elaphus (Red deer)	*S. putrefaciens* AS006C2	AML,AMC, IPM, KF, FOS, E	-

6.3.3 Minimal inhibitory concentration (MIC) of ciprofloxacin (CIP) and imipenem (IPM)

The obtained MIC values of CIP and IPM for *Acinetobacter* spp. AS027A3a, *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1 and *S. putrefaciens* AS006C2, are summarized in Table 6.2. *K. pneumoniae* AS027A2 was the least sensitive strains to the action of CIP with a MIC of 60 µg/ mL. *S. putrefaciens* AS006C2 was the most resistant strain to IPM with a MIC of 12 µg/ mL. However, IPM was the most efficient antibiotic against the four strains (12 µg/ mL to *S. putrefaciens* AS006C2; 6 µg/ mL to *P. fluorescens* AS008A1, 6 µg/ mL to *K. pneumoniae* AS027A2 and 2 µg/ mL to *Acinetobacter* spp. AS027A3a). According to the CLSI (2014) guidelines all strains are considered resistant to CIP (MIC > 4 µg/ mL).

Table 6.2 MIC of CIP and IPM against *Acinetobacter* spp. AS027A3a, *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1 and *S. putrefaciens* AS006C2

Strains	MIC (µg/ mL)	
	CIP	IPM
Acinetobacter spp. AS027A3a	44	2
K. pneumoniae AS027A2	60	6
P. fluorescens AS008A1	6	6
S. putrefaciens AS006C2	24	12

Strains *K. pneumonia* AS027A2, *P. fluorescens* AS008A1 and *S. putrefaciens* AS006C2 also are considered resistant to IMP (MICs > 4 µg/ mL). While *Acinetobacter* spp. AS027A3a is classified as intermediate (MIC = 2 µg/ mL)(CLSI, 2014).

6.3.4 Bacterial surface hydrophobicity

All the bacteria tested had a hydrophilic surfaces (ΔG_{bwb}^{TOT}), being the values obtained significantly different between the selected strains ($P < 0.05$). Based on the Lifshitz-van der Waals (Υ^{LW}) component (Table 6.3) of surface tension parameters, all the strains are predominantly electron donor (Υ^{-}). *K. pneumonia* AS027A2 and *Acinetobacter* sp. AS027A3a had similar electron donor capacity ($P > 0.05$). *P. fluorescens* AS008A1 have only an electron donating character (Υ^{+}=0 mJ/ m^2). *S. putrefaciens* AS006C2 have the highest value of polar component polar component (Υ^{AB}) ($P > 0.05$).

Table 6.3 Surface tension parameters (Υ^{LW}, Υ^{AB}, Υ^{+}, Υ^{-}); and hydrophobicity (mJ/ cm^2) ΔG_{bwb}^{TOT}. Values are means ± SDs of three independent experiments

Animal species names	Bacteria	Surface tension parameters (mJ/ cm^2)			Hydrophobicity (mJ/ cm^2) ΔG_{bwb}^{TOT}
		Υ^{AB}	Υ^{+}	Υ^{-}	
R. americana (Greater rhea)	*Acinetobacter* spp. AS027A3a	12.6±0.6	0.7±0.1	54.4±0.6	33.7±1.0
	K. pneumoniae AS027A2	19.3±0.4	1.8±0.1	52.1±0.2	28.1±0.2
V. vulpes (Fox)	*P. fluorescens* AS008A1	0.0±0.0	0.0±0.0	69.8±0.1	60.2±0.1
C. elaphus (Red deer)	*S. putrefaciens* AS006C2	26±0.6	3.7±0.0	47.9±0.0	22.4±0.0

6.3.5 Initial monolayer adhesion

Figure 6.2 shows the ability of the strains to adhere to PS surface. The degree of bacterial attachment has been found to follow the sequence: *P. fluorescens* AS008A1> *S. putrefaciens* AS006C2 > *K. pneumoniae* AS027A2> *Acinetobacter* spp. AS027A3a (Figure 6.2).

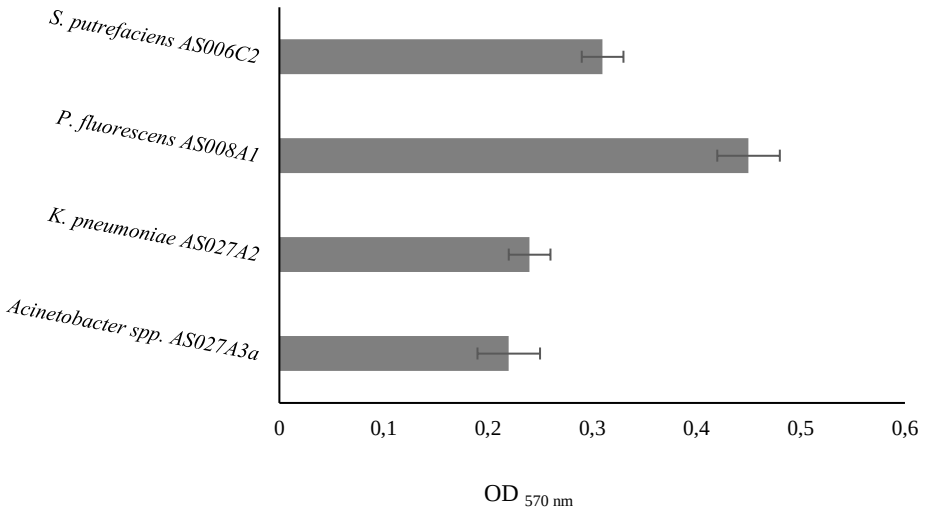

Figure 6.2 Values of OD $_{570\ nm}$ as a measure of bacterial adhesion on PS during 2 h. Mean values ± SDs for three independent experiments are illustrated.

S. putrefaciens AS006C2, *K. pneumoniae* AS027A2 and *Acinetobacter* spp. AS027A3a were classified as moderately adherent while *P. fluorescens* AS008A1 was classified as strongly adherent (Table 6.4). *P. fluorescens* AS008A1 and *Acinetobacter* spp. had the highest and lowest adhesion abilities, respectively. *K. pneumoniae* AS027A2 and *Acinetobacter* spp. AS027A3a adhered at similar extents ($P > 0.05$).

Table 6.4 Adhesion and biofilm formation ability of the four strains according to the classification proposed by Stepanović *et al.* (2000)

Animal species names	Species	Adhesion	Biofilm		
			24 h	48 h	72 h
R. americana (Greater rhea)	*Acinetobacter* spp. AS027A3a	++	++	++	++
	K. pneumoniae AS27A2	++	++	+++	+++
V. vulpes (Fox)	*P. fluorescens* AS008A1	+++	+	+	+
C. elaphus (Red deer)	*S. putrefaciens* AS006C2	++	+	++	++

Note: (0) Non-adherent/non-biofilm producer; (+) weakly adherent/ weak biofilm producer; (++) moderately adherent/moderate biofilm producer; (+++) strongly adherent/strong biofilm produce.

6.3.6 Biofilm formation

The strains were studied for biofilm formation ability in 96-well polystyrene microtiter plates during 24, 48 and 72 h. Figure 6.3 shows that all strains are able to form biofilms for all the sampling times. *P. fluorescens* AS008A1 produced the smallest biomass amount for the three sampling times. On the other hand, *K. pneumoniae* AS027A2 produced the highest biomass amount. The amount of biofilm produced was directly proportional to the biofilm age for *Acinetobacter* spp. AS027A3a and *S. putrefaciens* AS006C2. While for *P. fluorescens* the biomass increased from 24 to 48 h and remained constant for the 72 h old biofilms. *K. pneumoniae* AS027A2 developed weak biofilm during the first 24 h. The biofilm formation ability increased at 48 h and decreased at 72 h. It was found that the degree of biofilm formation followed the sequence: 24 h-aged biofilms: *K. pneumoniae* AS027A2 > *Acinetobacter* spp. AS027A3a > *S. putrefaciens* AS006C2 and *P. fluorescens* AS008A1; 48 h and 72 h-aged biofilms: *K. pneumoniae* AS027A2 > *Acinetobacter* spp. AS027A3a > *S. putrefaciens* AS006C2 > *P. fluorescens* AS008A1. According to the classification proposed by Stepanović *et al.* (2000), concerning the capability of the bacteria to form biofilms (Table 6.4).

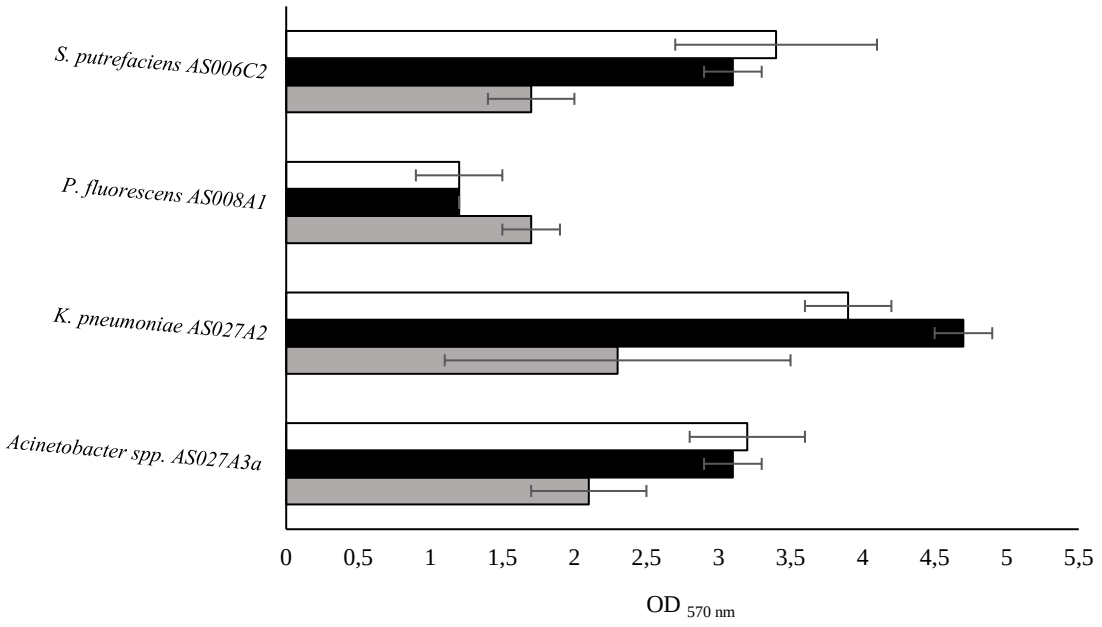

Figure 6.3 Values of OD $_{570\ nm}$ as a measure of mass of 24 h (▒), 48 h (■) and 72 h (□) of growth in biofilms. Mean values ± SDs for three independent experiments are illustrated.

P. fluorescens AS008A1 and *Acinetobacter* spp. AS027A3a showed a weak and moderate biofilm production for the various sampling times, respectively. *S. putrefaciens* AS006C2 presented a weak biofilm formation ability at 24 h and moderate biofilm formation ability after 48 h and 72 h. *K. pneumoniae* AS027A2 demonstrated moderate ability to form biofilm after 24 h and presented strong biofilm formation ability at 48 h and 72 h.

6.3.7 Effect of ciprofloxacin (CIP) and imipenem (IPM) on biofilm removal

The effect of the IPM and CIP was tested on the removal of 24 h old biofilms of *S. putrefaciens* AS006C2, *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1 and *Acinetobacter* spp. AS027A3a (Figure 6.4). In general, the increase of the concentration CIP or IPM increase biofilm removed.

A significant increase of the percentage of *P. fluorescens* AS008A1 biomass reduction occurred with CIP from MIC to 5× MIC, however, the duplication to 10×MIC resulted only in a small increase of the biomass reduction percentage. A similar behavior was observed for *K. pneumoniae* AS027A2 with IPM.

For *S. putrefaciens* AS006C2 the two highest IPM concentrations evaluated (5× MIC and 10× MIC) showed the same percentage of biofilm mass reduction (36%). No biofilm mass removal was obtained with IPM at MIC against *S. putrefaciens* AS006C2, *P. fluorescens* AS008A1 and *Acinetobacter* spp. AS027A3a, and with CIP at MIC and 5× MIC against *Acinetobacter* spp. AS027A3a. IPM at 10× MIC caused the highest reductions of *S. putrefaciens* AS006C2 (36% removal) and *K. pneumoniae* AS027A2 (54% removal) biofilm mass removal. CIP at 10× MIC caused the highest biomass removal of *P. fluorescens* AS008A1 biofilms (40%). *Acinetobacter* spp. AS027A3a biofilms were highly resistance to removal by CIP and IPM (a maximum removal of 10% was obtained with IPM at 10× MIC). In fact, *Acinetobacter* spp. AS027A3a biofilms were the most resistant to CIP while *P. fluorescens* AS008A1 biofilms were the most resistant to IPM.

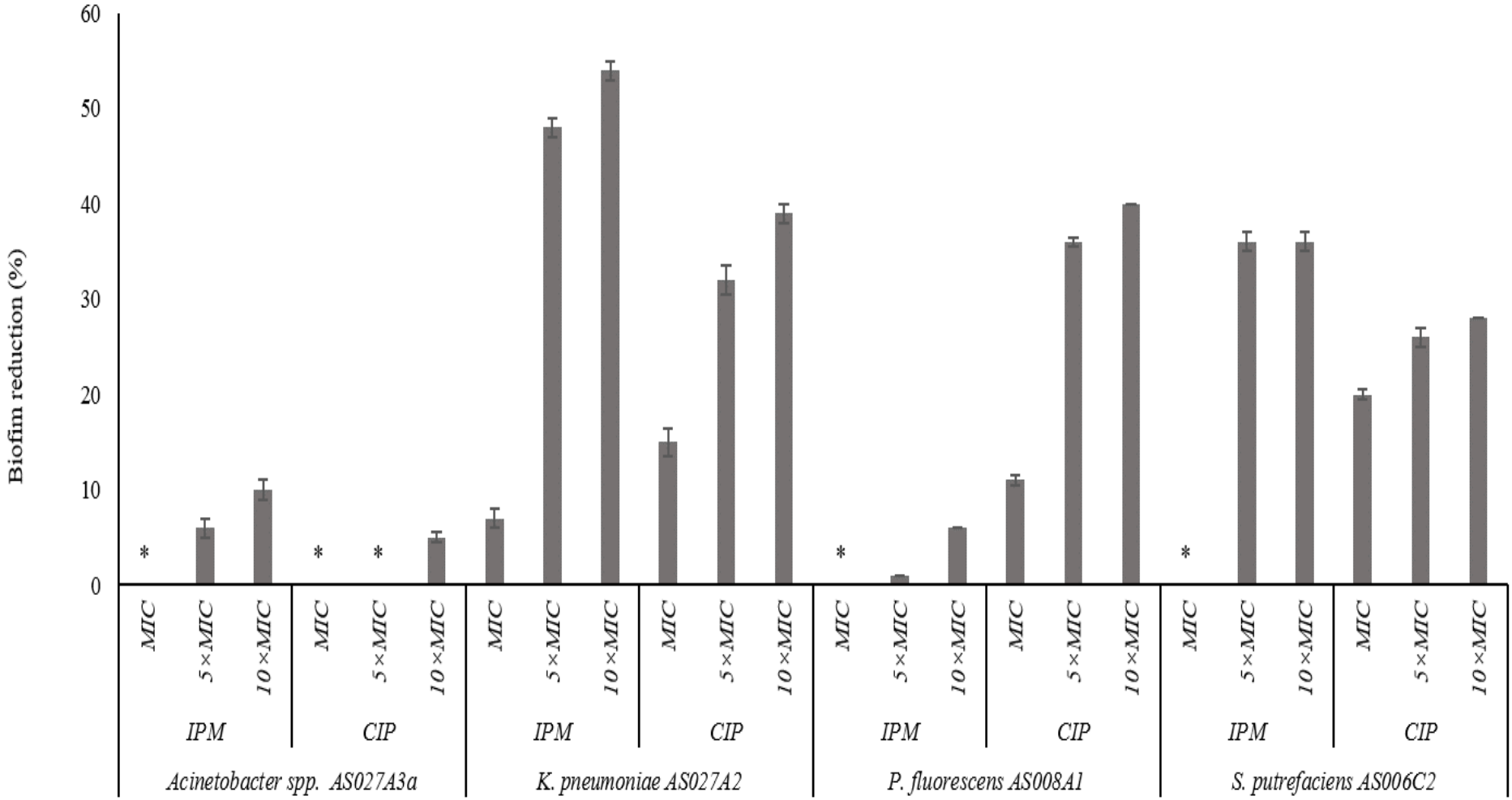

Figure 6.4 Percentage of mass reduction of *S. putrefaciens* $_{AS006C2}$, *P. fluorescens* $_{AS008A1}$, *K. pneumoniae* $_{AS027A2}$ and *Acinetobacter* spp. $_{AS027A3a}$ 24 h-old biofilms treated with IPM and CIP (at MIC, 5× MIC and 10× MIC) for 24 h. * No biofilm mass reduction was found. Mean values ± SDs for at least three replicates are illustrated.

6.4 DISCUSSION

The emergence of multiresistant microorganisms remains a serious global health concern (da Costa *et al.*, 2013). The gut of humans and animals are one important reservoir of multiresistant bacteria (Wellington *et al.*, 2013). In general, most of the microbiological studies have been focused on the gut of human and domestics/farm animals. However, the role of the wild animals as a reservoir of antibiotic resistant bacteria has been acquiring attention in the last years (Sjolund *et al.*, 2008, Hernandez *et al.*, 2012, Finley *et al.*, 2013, Smith *et al.*, 2014). In the present study, multiresistant strains were obtained from the fecal sample of wild animals of the north of Portugal. Based on the identification results by 16S rRNA gene sequencing the selected strains belong to four distinct genus: *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1, *S. putrefaciens* AS0062 and *Acinetobacter* spp. AS027A3a. *K. pneumoniae* AS027A2 and *Acinetobacter* spp. AS027A3a are important opportunistic pathogens due to the rapid increase of resistant to most of the currently available antibiotics, particularly to carbapenems (Poireland Nordmann, 2006, Snitkin *et al.*, 2012). *S. putrefaciens* AS006C2 can be widely found in the nature and especially in marine environments. In some cases *S. putrefaciens* AS006C2 can become pathogenic for humans and produce a wide variety of clinical syndromes including bacteremia (Patel *et al.*, 2012). *P. fluorescens* AS008A1 has generally been regarded to be of low virulence and an infrequent cause of human infections. However, it has been associated with some infections such as blood transfusion-related septicemia, catheter-related bacteremia, and peritonitis in peritoneal dialysis patients (Wong *et al.*, 2011).

The occurrence of multidrug-resistant bacteria in wild animals that live in extremes and remote environments with low contact with antimicrobials or even never exposed to them has been previously recognized (Hernandez *et al.*, 2012, Smith *et al.*, 2014). For instance, studies conducted by Simões *et al.* (2012) reported multiresistant *Escherichia coli* in faeces of Iberian wolves (*Canis lupus signatus*). The presence of multiresistant bacteria in the wild animals represent a public health concern due to the increased occurrence of zoonotic diseases that can be disseminated to people or domestic animals as well as the need to predict emerging resistant pathogens (Taylor *et al.*, 2001). Two features that are frequently understated are the mechanisms of resistance to antibiotics and its dissemination. Another important aspect is that, in many cases, the

classes of antibiotics used in human and veterinary medicine are the same (Li *et al.*, 2007).

Resistance to multiple antibiotics was observed by the strains of the present study. Moreover, the strains showed resistance to IPM, a *β*-lactam antibiotic typically used for the treatment of complex multiresistant human infections. *β*-lactam antibiotics are an important group of broad-spectrum antibiotics used in both human and animal health for the treatment of bacterial infections (Li *et al.*, 2007). The intensive use of these antibiotics has contributed to the emergence of resistant bacteria, including bacteria of animal origin (Li *et al.*, 2007).

The production of *β*-lactamases, a family of enzymes that hydrolyzes the *β*-lactam ring, thereby inactivating the antibiotic molecule prior to binding with Penicillin Binding Proteins (PBP`s), is the principal mechanism of resistance to *β*-lactam antibiotics. They also play a major role in the intrinsic and acquired resistance in bacteria, principally in Gram-negative bacteria (Li *et al.*, 2007). In order to investigate which are the genes conferring resistance to *β*-lactam antibiotics, several genetic determinants were amplified by PCR. These results revealed the presence of *β*-lactamases in two strains *P. fluorescens* AS008A1 and *K. pneumoniae* AS027A2. In addition, it was possible to observe that extended-spectrum *β*-lactamase-producing (ESBL`s) *K. pneumoniae* AS027A2 strain carries multiple *β*-lactamase genes: $bla_{\text{OXA-aer}}$, bla_{TEM} and bla_{SHV} genes. In the *P. fluorescens* AS008A1 strain was only detected the $bla_{\text{OXA-aer}}$ gene. ESBL as well as TEM and SHV type producing bacteria are frequently present in the gastrointestinal tract of animals (Carattoli, 2008, Coque *et al.*, 2008) and have been detected from various animals, including wildlife (Patersonand Bonomo, 2005, Carattoli, 2008). It is important to note that there are a large number of *β*-lactamases and ESBLs resistant to *β*-lactamase inhibitors, which have been derived from TEM and SHV enzymes as a consequence of amino acid substitution in their sequences (Bradford, 2001). In the *P. fluorescens* AS008A1 strain only the $bla_{\text{OXA-aer}}$ gene was detected. For *S. putrefaciens* AS006C2 and *Acinetobacter* spp. AS027A3a strains the presence of the studied *β*-lactamases genes was not detected. Therefore, it can be hypothesized that in these strains the resistance to *β*-lactam antibiotics can be mediated by other mechanisms, namely the decrease of the intracellular concentration of the antibiotic as a result of poor penetration into the bacterium or the presence of efflux pumps, and the alteration of the antibiotic target by post-translational modification of the target or genetic mutation (Li *et al.*, 2007).

However, the results of this study can support the hypothesis that wild animals constitute antibiotic resistance reservoirs.

During the course of their evolution, bacteria have continuously modified their metabolism and physical characteristics, adapting to almost all environments (Costerton *et al.*, 1995, Hoiby *et al.*, 2010).

In order to survive in hostile environments such as that encountered in the host tissues, bacteria have adapted to exist as communities of adhered cells (Mahand O'Toole, 2001). A direct relationship between the occurrence of microorganisms as sessile communities and the infectious diseases has been reported in animals (de la Fuente-Nunez *et al.*, 2013). According to studies conducted by Bakker et al. (2004) bacterial pathogenic strains obtained from different niches can exhibit different abilities to adhere to a surface. In the present study, all the strains had the ability to adhere on PS and form biofilms. However, with distinct magnitudes. *P. fluorescens* AS008A1 was the strain with the highest ability of adhesion. However, it demonstrated low ability to form biofilm. *K. pneumoniae* AS027A2 was the strain with the highest ability to form biofilm. It was also the strain more resistant to CIP and carrying multiple types of *β*-lactamase genes. Hennequin *et al.* (2012) recognised the influence of capsule and ESBLs encoding plasmids upon *K. pneumoniae* adhesion. Another work developed by Yang and Zhang (2008) with *K. pneumoniae* strains, isolated from sputum and urine, demonstrated the existence of a relationship between the ability to form biofilm and the production of ESBL. These observations corroborate the results obtained in the present study, as the strain that express more *β*-lactamase genes was the most proficient to produce biofilm.

Recent evidences on the ability of wild animal strains to form biofilms and recent data supporting the correlation of such behaviour with antibiotic resistance acquisition, alert even more for the hazard of these strains to human health (Schillaciand Vitale, 2012). Strains such as *K. pneumoniae* AS027A2 can form biofilms in the gastrointestinal tract and natural cavities of humans (Chen *et al.*, 2014). In biofilms the bacteria are more resistant to the activity of the antibiotics and host defence mechanisms due to the presence of one polysaccharide matrix, low growth rate and the expression of possible biofilms specific resistance genes (Stewartand Costerton, 2001, Stewart, 2002, Taylor *et al.*, 2011). Biofilms also can be important to the spread of antibiotic resistance by promoting horizontal genes transfer (Fux *et al.*, 2005). This aspect represents important difficulties in the treatment of biofilm infections in human and animal organisms (Fux *et al.*, 2005). In the present study, the capacity of CIP and IPM (MIC; 5× MIC; 10× MIC) to remove

biofilms was evaluated. Both IPM and CIP are antibiotics commonly used to control bacterial infection, with a wide antibacterial spectrum (Tamma *et al.*, 2012). In general, the percentage of biofilm mass reduction was lower than 55%.

For three of the strains (*S. putrefaciens* $_{AS006C2}$, *P. fluorescens* $_{AS008A1}$ and *Acinetobacter* spp. $_{AS027A3a}$) tested the two antibiotics had no activity at MIC. *Acinetobacter* spp. $_{AS027A3a}$ biofilm was the more resistant to CIP antibiotic. These high levels of resistance to CIP in clinic strains of *Acinetobacter* spp. $_{AS027A23a}$ also have been later reported by (Abdi-Ali *et al.*, 2014). IPM was the most active antibiotic since it significantly reduced the mass of *S. putrefaciens* $_{AS006C2}$ and *K. pneumoniae* $_{AS027A2}$ biofilms.

6.5 CONCLUSION

The existing levels of resistance to antibiotic in wild animals can create a continuous selective pressure on β-lactamases that is critical to public as well as animal health. The analysis of the phenotypic and genotypic resistance profile of the strains studied in this work demonstrated that they are multiresistant and possess antibiotic resistance determinants. The biofilm forming ability further reveals the resilience and antibiotic resistance of strains used in this study. Moreover, the data highlight that efforts should be implemented in order to monitor and control the use of antibiotics and their dissemination in the environment. In addition, regular monitoring of the multiresistant and proper assessment of the antimicrobial resistance mechanisms among bacteria of animal origin could help to minimize the selective pressure and improve the treatment strategies in both humans and animals.

CHAPTER 7. ASSESSMENT OF THE VIABILITY OF STRAINS ISOLATED FROM WILD ANIMAL UNDER SIMULATED HUMAN GASTROINTESTINAL TRACT CONDITIONS

ABSTRACT

Infectious diseases are commonly preceded by gastrointestinal colonization, and the gastrointestinal tract represents the most important reservoir for transmission of bacteria. In the last years, the wild animal reservoirs have been implicated as a source of multiresistant bacteria responsible for human diseases. Understanding human-animal association plays a key role in the emerging infectious diseases dissemination. This study intends to evaluate the viability of six strains (*Acinetobacter* spp. AS027A3a, *A. salmonicida* AS006C3c1, *A. veronii* AS070GSP1, *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1 and *S. putrefaciens* AS006C2) isolated from different wild animals to colonize the human gastrointestinal tract, using a static monocompartmental system with an artificial gastrointestinal medium. It was be observed, that the gastrointestinal tract significantly reduced bacterial viable counts. However, two strains, *A. salmonicida* AS006C3c1 and *K. pneumoniae* AS027A2, were able to survive under such conditions.

Keywords: Gastrointestinal tract, growth curves, pH, wild animals

7.1 INTRODUCTION

Bacteria are important elements of the biosphere, and their presence invariable affects the environment that they are growing in. The effects of bacteria on the environment can be beneficial or harmful, or imperceptible with regard to human measure or observation (Merchantand Helmann, 2012).

In the last years, several infectious diseases have emerged with often devastating consequences for global health and impacts on trade and economies. The emergence and spread of antibiotic resistant bacteria are worldwide one of the most important public health problems (Fonkwo, 2008).

Some studies have demonstrated that more than 70% of emerging infectious diseases in the past two decades are zoonotic in origin. Wildlife animals are considered one of the major reservoirs for transmission of zoonotic agents to humans. Nowadays, zoonotic infectious diseases with wild animal reservoirs are increasingly recognized, and need for more attention from a public health perspective (Kruse *et al.*, 2004, Cutler *et al.*, 2010).

Understanding human-animal association plays a key role in emerging infectious diseases (Cleaveland *et al.*, 2001, VanderWaal *et al.*, 2014). The epidemiology of multiresistant bacteria at the human/animal interfaces involves complex and largely unpredictable systems, which involve transmission routes of antibiotic resistant bacteria as well as resistant genes, and the impact of antibiotic selective pressures in several reservoirs include animals, humans and the environment (Baquero *et al.*, 2011).

The human gastrointestinal tract is the most densely inhabited ecosystems on Earth. The human gastrointestinal tract is colonized by a complex and dynamic, but stable, community of bacterial species referred to as the gut microbiota, which has numerous important roles in human physiology (Payne *et al.*, 2012).

Gastrointestinal bacterial communities play a determining role on protective, structural and metabolic function in host health (Williams *et al.*, 2015). This includes digestion of nutrients, protection against invading pathogens a process known as homeostasis, and immune enhancement (Probertand Gibson, 2002, Macfarlane *et al.*, 2004, Macfarlaneand Macfarlane, 2004).

The contribution of the gut microbiota to host health has been well study with dysbiosis being linked to several disease states including diabetes, obesity inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). Modulation of the composition and metabolic

activity of these microbiota to improve health attracts a lot of attention and is referred to as gut resource management (Gerritsen *et al.*, 2011).

Numerous *in vivo* and *in vitro* experimental studies have been used to study the response of gastrointestinal microorganisms to several different perturbations or treatments. However, due to the human gastrointestinal tract physically inaccessible for sampling, ethical restrictions implicated in human trial, as well as several variable compliance, a substantial research has been done to develop *in vitro*, *ex vivo*, *in silico*, and animal models to understand the human gastrointestinal tract (Macfarlaneand Macfarlane, 2007).

Different types of *in vitro* systems with varied complexity and applicability have been developed, ranging from simple nonstirred batch cultures without pH control, to more complex continuous systems involving pH, controlled single or dynamic multicomportment cultures models. These *in vitro* models provide a quick, easy, and cost-effective means of studying the gastrointestinal microbiota, in one or more gut segments (Williams *et al.*, 2015).

This study intends to evaluate the viability of five strains (*Acinetobacter*, *Aeromonas*, *klebsiella*, *Pseudomonas* and *Shewanella*), isolated from different wild animals (birds and mammals) under simulated human gastrointestinal tract conditions.

7.2 MATERIAL AND METHODS

7.2.1 Culture conditions and bacterial strains

The strains used in this study were *Acinetobacter* spp. AS027A3a, *A. salmonicida* AS006C3c1, *A. veronii* AS070GSP1, *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1 and *S. putrefaciens* AS006C2. The strains were obtained from overnight cultures in 250 mL flasks with 100 mL of TSB (Merck, Germany) at 37 ºC and under agitation (150 rpm).

7.2.2 Static monocompartmental models

A batch cultures system was used for this work. A batch cultures constitute the simplest forms of *in vitro* systems used to study the human gut microbiota. The gastrointestinal environment was simulated based on previous studies of Abadía-García *et al.* (2013) and Guerra *et al.* (2012), according to the procedure presented in sub-section 3.14.

7.2.3 Planktonic growth curves

Growth curves were determined for the strains that showed ability to survive under simulated human gastrointestinal conditions using a FLUOstarOPTIMA (BMGLabtech, U. K.), according to the procedure described in sub-section 3.15. At least three independent experiments were performed.

7.3 RESULTS

7.3.1 Static monocompartmental system

In order to examine the ability of the six strains to growth under human gastrointestinal tract conditions CFU/ mL were assessed after 30, 60, 120, 150, 180, 240, 300 and 360 min. The strains used in this study were selected because they presented multiresistance to antibiotics, four of which presented resistance to imipenem (*Acinetobacter* spp. AS027A3a, *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1 and *S. putrefaciens* AS006C2) an antibiotic belonging to an important class of antibiotics used only in human medicine (*β*-lactam - carbapenem).

Initially the strains were placed in an artificial medium of saliva with pH 7. Subsequent passage through simulated human gastrointestinal tract condition, with artificial medium with pH adjusted to 4, showed that the influence of gastrointestinal tract significantly reduced viable counts. However, two strains, *A. salmonicida* AS006C3c1 and *K. pneumoniae* AS027A2, were capable to survive under the simulated human gastrointestinal tract conditions (Figure 7.1).

From the analysis of Figure 7.1, in general, it can be observed that the number of CFU/ mL of the two strains increased over time, however, the number did not change significantly during the experiment. for both strains, the most significant increase in cells numbers occurred between 240 and 300 min ($P < 0.05$) (*A. salmonicida* AS006C3c1 – nº CFU/ mL = 8.63±0.47; *K. pneumoniae* AS027A2 - nº CFU/ mL = 8.30±0.01).

In the first 90 minutes, *A. salmonicida* AS006C3c1 was the strain with the highest number of cells ($P < 0.05$). This difference decreases over time. However, after 300 min the number of CFU/ mL of *A. salmonicida* AS006C3c1 decreased, while the number of CFU/ mL of *K. pneumoniae* AS027A2 increased moderately and not statistically significant ($P > 0.05$).

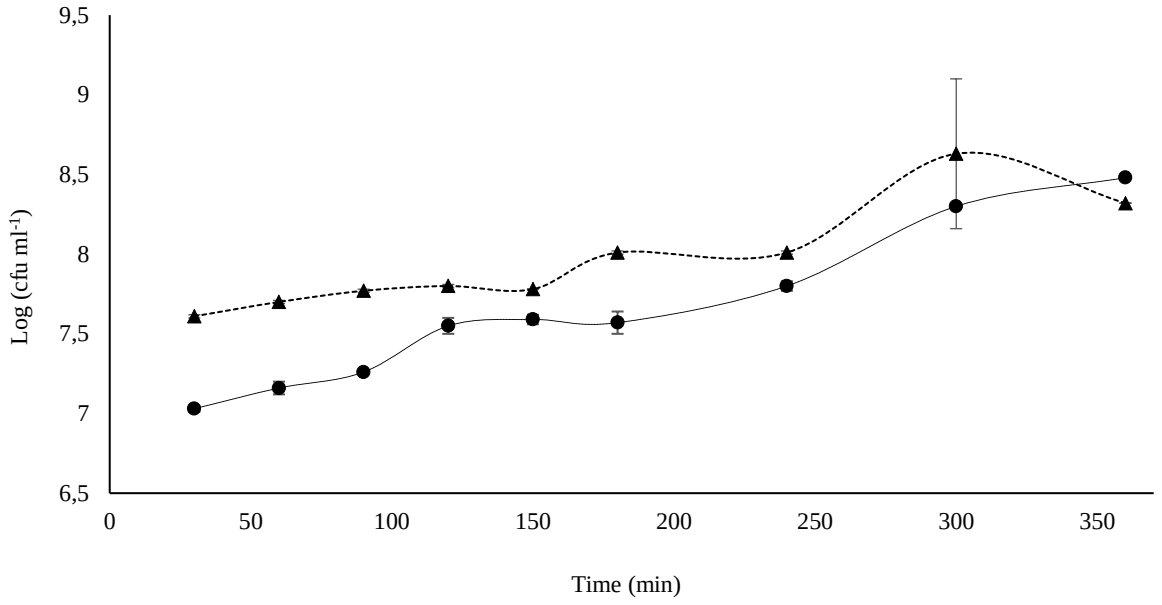

Figure 7.1 Log CFU/ mL for *A. salmonicida* $_{AS006C3c1}$ (---▲---) and *K. pneumoniae* $_{AS027A2}$ (--●--) after 350 minutes under human gastrointestinal conditions. The means ± SD for least three replicates are illustrated.

7.3.2 Planktonic growth curves

The growth rates of the two strains that survive in simulated gastrointestinal tract conditions were determined to see which strains showed the best ability to survive. Optical densities in 96-well plates were measured every 30 min, in the first five hours and in 60 min intervals for the remaining 43 h.

Figure 7.2 shows the growth curves of the two strains, all three growth phases of strains are clearly defined from each other. The lenght of lag phase was longer in *K. pneumoniae* $_{AS027A2}$ (9 h) than *A. salmonicida* $_{AS006C3c1}$ (5 h).

The analysis of the growth profiles shows that *A. salmonicida* $_{AS006C3c1}$ had a longer exponential phase, starting the stationary phase 28 h after incubation. *K. pneumoniae* $_{AS027A2}$ achieved its stationary phase 30 h after incubation.

A growth decline phase started earlier for *A. salmonicida* $_{AS006C3c1}$ (29 h after incubation) than for *K. pneumoniae* $_{AS027A2}$ (39 h after incubation).

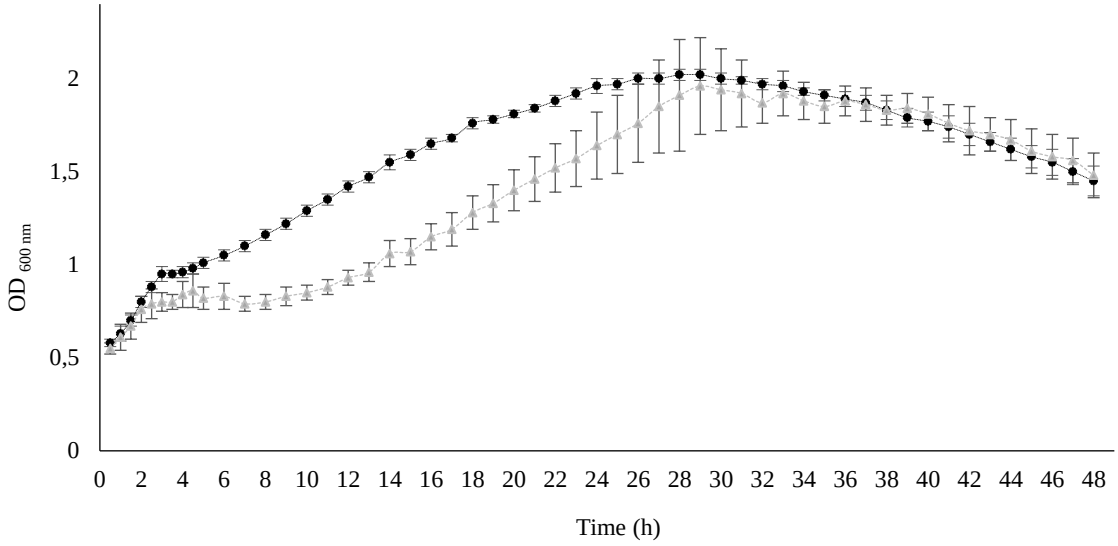

Figure 7.2 Growth curves of *A. salmonicida* AS006C3c1 (—●—) and *K. pneumoniae* AS027A2 (- -▲- -) at 37 ºC and pH 4. The means ± SD for a least three replicates are illustrated.

Table 7.1 shows the specific growth rate as well as doubling time for each strain.

Table 7.1 Specific growth parameter (specific growth rate - μ (h-1) and doubling time - T_d (h)) of *A. salmonicida* AS006C3c1 and *K. pneumoniae* AS027A2 at 37 ºC and pH 4

Strain	μ (h^{-1})	T_d (h)
A. salmonicida AS006C3c1	0.046±0.002	13.0±0.5
K. pneumoniae AS027A2	0.053±0.002	15.1±0.7

A. salmonicida AS006C3c1 exhibited a slower specific growth rate than *K. pneumoniae* AS027A2, but not very significant ($P < 0.05$). Consequently, *A. salmonicida* AS006C3c1 had a doubling time higher than *K. pneumoniae* AS027A2.

7.4 DISCUSSION

The aim of this study was the assessment of the viability of six strains (*A. salmonicida* AS006C3c1, *A. veronii* AS070GSP1, *Acinetobacter* spp. AS027A3a, *K. pneumoniae* AS027A2, *P. fluorescens* AS008A1 and *S. putrefaciens* AS006C2) isolated from different wild animals under simulated human gastrointestinal tract conditions.

It is recognized that animals depend on their associated bacterial communities to help with digestion, immunity, and other aspects of physiology and disease (Hehemann *et al.*, 2010, Wang *et al.*, 2011, Eloe-Fadroshand Rasko, 2013). The gastric pH is considered to be an effective barrier for bacterial growth. However, several pathogens have developed multiple resistance mechanisms to increase their survival in the human gastrointestinal tract (O'May *et al.*, 2005). To understand the structure and dynamics of the bacterial community in the gastrointestinal tract several researches studied the response of bacterial communities to variations in diet, health or environment (Maurice *et al.*, 2013, David *et al.*, 2014). For example, interpopulation comparisons, such as between Italian and Burkino Fasan children or between Americans, Malawians, and Amerindians, measured gut microbiome differences that are likely associated with differences in climates, genetic background, access to medical care, sanitary practices, and pathogen exposure across populations, in addition to differences in diet (Mai *et al.*, 2009, De Filippo *et al.*, 2010, Yatsunenko *et al.*, 2012, Davenport *et al.*, 2014).

In this study the six strains were inoculated in batch systems with TSB medium supplemented with pepsin from porcine gastric mucosa and the pH was adjusted to 4 and 37 ºC, after they remain in artificial saliva medium (pH 7), in order to simulate the human gastrointestinal system conditions. It was possible to observe that two strains, *A. salmonicida* AS006C3c1 and *K. pneumoniae* AS027A2, were able to survive under simulated human gastrointestinal conditions. *A. salmonicida* and *K. pneumoniae* are Gram-negative species usually associated with gastrointestinal diseases (Maroncle *et al.*, 2002, Ghenghesh *et al.*, 2008). *A. salmonicida* is the etiological agent of furunculosis, an infectious disease characterized by the presence of haemorrhagic and necrotic lesions in the gills, gut and muscle. Insonhood and Dranke (2002) demonstrates that *Aeromonas* species can resist pH range from 4 to 9, but the optimum pH range from 5.5 to 9. The results of the present work are in agreement with the works described by Insonhood and Dranke (2002) since in this study the strains can survive in a culture medium at pH 4.

K. pneumoniae is an opportunistic pathogen, encountered as saprophyte in humans and other animals, colonizing the gastrointestinal tract, skin, and nasopharynx; it is also found in different environmental niches (Struveand Krogfelt, 2004). Epidemiological studies have shown that *K. pneumoniae* infections are frequently preceded by gastrointestinal colonization, in the present study it can also be demonstrated that *K pneumoniae* AS027A2 isolated from wild animal can survive under simulated human gastrointestinal tract conditions. It is worthy to not that that strain showed a multiresistance phenotype and harboured one OXA, TEM and SHV. The prevalence of carbapenem-resistant *K. pneumoniae* has been increasing globally, making

antimicrobial treatment difficult and causing higher disease-related mortality rates (Nordmann *et al.*, 2009, Neuner *et al.*, 2011). The virulence factors playing an important role in the severity of *K. pneumoniae* infectious diseases are capsular polysaccharides, factors involved in aggregative adhesions and siderophore production (Vuotto *et al.*, 2014). In a study carried out by (Podschun *et al.*, 2001) it was established that a *K. pneumoniae* strain from waters resemble clinical strains in the expression of virulence factors, suggesting that environmental strains of *K. pneumoniae* may be as virulent as clinical strains.

7.5 CONCLUSION

In conclusion, with this study, it can be observe that strains isolated from different wild animals can survive in the human gastrointestinal tract, once they can survive under simulated human gastrointestinal conditions, and may represent a public health problem. Due to their resistance phenotype and their biofilm forming ability, the treatment of the infectious diseases caused by these strains can be difficult, and requires high economic costs to human medicine. Nonetheless, an opportunity now exists to understand the importance of wild animals to human health, into wild animal species could result in wildlife hosts functioning as a reservoirs and vectors for reintroduction of pathogenic microorganism to the human population.

CHAPTER 8. VIRULENCE ASPECTS OF BACTERIA ISOLATED FROM WILD ANIMALS INCLUDING THEIR ABILITY TO INVADE HUMAN COLON ADENOCARCINOMA CELLS LINE CACO-2

DIAS, C., BRANCO, A., DOMÍNGUEZ-PERLES, R., MARTEL, F., SAAVEDRA, M.J., SIMÕES, M. Biofilm and ability to invade Human colon adenocarcinoma Caco-2 cell line of bacteria isolated from wild animals. (*In preparation*)

ABSTRACT

The emergence of multiresistant bacteria has accelerated in the last years, mainly as a result of selective pressure from human activities. The concept "One Health" was constituted to attempt to monitor and control the risks of antibiotics resistance dissemination between humans, animals and the environment. The pathogenesis of infectious diseases is therefore complex and multifactorial. Cell-cell interaction is an important first step in bacterial pathogenesis. In this work, six different strains (*S. putrefaciens* AS006C2, *P. fluorescens* AS008A1, *K. pneumoniae* AS027A2, *Acinetobacter* spp. AS0027A3a, *A. salmonicida* AS006C3c1, *A. veronii* AS070GSP1), isolated from wild animals were tested for their ability to produce *N*-acyl homoserine lactones (AHLs), gelatinases, proteases and siderophores, chemical molecules associated to virulence of the strains. The ability of the strains to attached/internalized to the human colonic adenocarcinoma cells line Caco-2 was also tested. Gelatinase ability was detected in all strains. *K. pneumoniae* AS027A2 was the only strain that produced siderophores, and *S. putrefaciens* AS006C2 was the only that did not express AHLs. All the strains were able to attach to Caco-2 cells and internalized. Among them *A. salmonicida* AS006C3c2 was the strain that presented the largest number of attached and internalized bacterial cells.

Keywords: Adhesion, bacteria, Caco-2 cell line, host cells invasion, wild animals

8.1 INTRODUCTION

The pathogenesis of infectious diseases caused by bacteria is a complex and multifactorial issue, whilst the most relevant factors have been extensively investigated upon assays of interaction between bacteria and cell cultures (Emody *et al.*, 2003). In this frame, antibiotic resistant bacteria are becoming more and more difficult to combat with available antibiotics (Dantas *et al.*, 2008). Indeed, frequency of multiresistant bacteria has augmented in the last years as a consequence of the abuse and mistaken administration of antibiotics in human and veterinary medicine, where concentration of active molecules and duration of the treatments are frequently not properly observed (Aarestrup, 2005). In this regard, antibiotic multiresistant bacteria is responsible by insidious infectious diseases responsible for high morbidity and mortality, as well as for increased treatment costs (Paul *et al.*, 2010).

In the last years, the awareness on this situation has prompted several organizations such as the European Centre for Disease Prevention and Control (ECDC), the US Centers for Disease Control and Prevention (CDC), and the World Health Organization (WHO) to organize a coordinated response against infectious diseases caused by multiresistant strains (Roca *et al.*, 2015). This constitutes a response to the current situation, where despite the several studies on the dissemination of bacteria strains resistant to clinical antibiotics; the scientific community remains largely ignorant on the complex dynamic of the dissemination of antibiotic resistant genes (Marti *et al.*, 2014).

From a biological point of view, antimicrobial resistance and virulence are features that help microorganisms to survive in adverse environments. Hence, while virulence is needed to outgrow host cell defense systems, antibiotic resistance is essential to enable bacteria to overcome medical therapies (Beceiro *et al.*, 2013). In this sense, antimicrobial resistance and virulence mechanisms are complementary mechanisms contributing to the microorganisms surveyor (Patel, 2005). However, the occurrence of multi-resistant bacteria in the environment is strongly associated to the incidence of infection diseases in humans or animals (Riesenfeld *et al.*, 2004, D'Costa *et al.*, 2006), being critical for the global dissemination of genes codifying for antibiotic resistance (da Costa *et al.*, 2013). In this frame, to control antibiotic resistance dissemination requires of the collaborative work of medical, veterinary, and environmental actors, working on epidemiological, diagnostic, and therapeutic issues (Asokan *et al.*, 2013).

Bacteria are present both inside and on the surface of the human body (skin and the mucous membranes). Most of these microorganisms are innocuous, some are beneficial, and some are even necessary. However, some strains categorized as pathogens, are devoted to colonize and damage the host, causing illness (Beceiro *et al.*, 2013). The most substantial reservoir microorganism is the gastrointestinal (GI) tract of animals and humans (Wellington *et al.*, 2013). Pathogenic strains have developed adaptive processes that enable them to epithelial cells functions, augmenting their penetration and causing diseases (Luand Walker, 2001). Adhesion of strains to intestinal epithelium is often recognized as a prerequisite for colonization of the human GI tract (Beachey, 1981, Finlayand Falkow, 1997). Many pathogenic bacteria are internalized by mammalian cells, constituting an important step in the infectious diseases that protects these strains from the immune response (Ribetand Cossart, 2015).

To date, many *in vitro* methods have been developed to study bacteria adhesion and invasion. Culture cell lines from the human intestinal tract are commonly used to investigate microbial adhesion and invasion (Angelisand Turco, 2011). Human colon adenocarcinoma cell line (Caco-2) is one of the most investigated cell line, because it shares morphological and functional features with normal small intestine cells in post-confluent stage *in vitro* (Haoand Lee, 2004). This fact has prompted to select Caco-2 cells as a useful model to replace human intestinal epithelial cells in the study of the adhesion and the invasion ability of enteric pathogens (Ferraretto *et al.*, 2007).

In this study, the interaction of six strains (*S. putrefaciens* AS006C2, *P. fluorescens* AS008A1, *K. pneumoniae* AS027A2, *Acinetobacter* spp. AS0027A3a, *A. salmonicida* AS006C3c1, *A. veronii* AS070GSP1) isolated from wild animals with the human colon adenocarcinoma cells line Caco-2, in order to investigate their invasion capacity (attached and internalized). Besides, this work pursued to shed some light on N-acyl homoserine lactones (AHL) signal molecule profile as well as on some virulence factors, including siderophores, proteases and gelatinases during the invasion process.

8.2 Material and Methods

8.2.1 Bacteria and growth conditions

Six strains were selected for this study: *S. putrefaciens* AS006C2, *P. fluorescens* AS008A1, *K. pneumoniae* AS027A2, *Acinetobacter* spp. AS0027A3a, *A. salmonicida* AS006C3c1 and

A. veronii $_{AS070GSP1}$. The strains were isolated originally from wild animals feces and were evaluated on their multiresistant properties (Sub-section 3.2).

The identification of the strains was performed by 16S rRNA and *gyr*B gene sequencing (Sub-section 3.1). The strains were selected because of their high resistance to antibiotics and their ability to form biofilms.

Half-concentrated Brain Heart Infusion medium (BHI, Oxoid, UK) was used to cultivate strains at 37 ºC with constant shaking (160 rpm).

8.2.2 *N*-acyl Homoserine Lactones (AHL) production

N-acyl homoserine lactones (AHLs) screening was performed using the *Chromobacterium violaceum* CV026 biosensor strain. The bacterial strains were cross-streaked with *C. violaceum* CV026. Purple pigmentation after 24 h indicated quorum sensing (QS) activity according with the previous descriptions available in the literature (McLean *et al.*, 2004) (Sub-section 3.6). Three independent assay were performed.

8.2.3 Production of siderophores, proteases and gelatinases

The strains were obtained from overnight cultures grown in 100 mL tubes with 50 mL TSB incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Göttingen, Germany). An $OD_{600\ nm}$ of 0.2 was obtained in a test tube with saline solution at 8.5 g/ L. The siderophores, proteases, and gelatinases tests were done according to the procedure described in sub-section 3.5.1, 3.5.2, and 3.5.3 respectively. At least three independent experiments were performed for all tests.

8.2.4 Caco-2 cells growth condition

The Caco-2 cells were obtained from the Deutsche Sammulung von Mikroorganismen and Zellkulturen (Braunschwieg, Germany). Cells between passages 20 and 25. Following 24 hours of incubation at 37 ºC and 5% CO_2, cultures were washed three times in phosphate buffer saline (PBS), and suspended in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen, USA). Afterward, Caco-2 cells, cultured in DMEM supplemented with 15% of fetal calf serum (FCS), 100 U/ mL penicillin, 25 mM HEPES, 100 µg/ mL streptomycin, and

0.25 μg/ mL amphotericin B, in 5% CO_2 atmosphere at 37 ºC (Sub-section 3.16.2) were infected with the target pathogen bacteria evaluated in the present work.

8.2.5 Bacterial infection of Caco-2 cells

Infection of human colon adenocarcinoma cells (Caco-2 cells) with the selected bacteria was carried out as described by (Edwardsand Massey, 2011). Bacteria from the diverse strains under evaluation attached or internalized to Caco-2 cells were assayed in 24-well microplates, according to the procedure described in sub-section 3.16.3. This procedure was performed in triplicate (n=3) and the results obtained were expressed in log of CFU/ mL.

8.2.6 Fluorescence microscopy

Fluorescence microscopy was done according to the description of the sub-section 3.17.

8.2.7 Statistical analysis

The results were analysed using the statistical program SPSS version 22.0, by analysis of variance (ANOVA). Statistical calculations were based on a confidence level ≥ 95% ($P < 0.05$ was considered statically significant).

8.3 RESULTS

8.3.1 Production of N-acyl homoserine lactones, siderophores, proteases, and gelatinases

According to information contained in Table 8.1 on gelatinases, siderophores, proteases, and (AHL) production (C4 - HSL; C6 - HSL), gelatinase activity was detected in all six strains assessed; although *A. veronii* AS070GSP1 was the strain showing the best production ability.

On the other hand, concerning siderophores, *K. pneumoniae* AS027A2 was the only strain capable to produce these high affinity iron-chelating compound. *S. putrefaciens* AS006C2, *P. fluorescens* AS008A1, *A. salmonicida* AS006C3c1 and *A. veronii* AS070GSP1 were competent on producing proteases, being *S. putrefaciens* AS006C2 the strain that exhibited the lowest

producing ability. *P. fluorescens* AS008A1 and *A. salmonicida* AS006C3c1 are the strains that presented a greater capacity to produce proteases, both with 10 mm of halo diameter. Finally, in which respect to the ability to synthesize *N*-acyl homocysteine lactone (AHL), this was only absent in *S. putrefaciens* AS006C2.

Table 8.1 Production of gelatinases, siderophores, proteases and N-Acyl homocysteine lactone (AHL) by the six strains isolated from wild animals

Strains	Gelatinase production	Siderophores Production	Proteases production	AHL production
S. putrefaciens AS006C2	+ (4 mm)	n.d.	5 mm	-
P. fluorescens AS008A1	+ (4 mm)	n.d.	10 mm	+
K. pneumoniae AS027A2	+ (4 mm)	+	0	+
Acinetobacter spp. AS027A3a	+ (4 mm)	n.d.	0	+
A. salmonicida AS006C3c1	+ (4 mm)	n.d.	10 mm	+
A. veronii AS070GSP1	+ (5 mm)	n.d.	9 mm	+

n.d. - not detected

8.3.2 Adhesion and internalization

In addition to the production of siderophores, proteases, and gelatinases, in this study, the six strains of bacteria strains under consideration (isolated from wild animals) were assessed on their capacity of adhesion and invasion to human colon adenocarcinoma cells, *in vitro*. The results evidenced that all six strains were able to attach and internalize into Caco-2 cells although with different ability (Figure 8.1).

When comparing the efficiency of the separate strains evaluated, it was noticed that *A. salmonicida* AS006C3c2 highest number of colony formation units of attached and internalized cells into Caco-2 cells (5.53 CFU/ mL, on average), while *S. putrefaciens* AS006C2 exhibited the lowest value (4.53 CFU/ mL, on average). *P. fluorescens* AS008A1, *Acinetobacter* spp. AS027A3a, *K. pneumoniae* AS027A2 and *A. veronii* AS070GSP1 showed intermediate levels of attached and internalized cell (5.0 CFU/ mL, on average), significantly lower and higher than *A. salmonicida* AS006C3c2 and *S. putrefaciens* AS006, respectively.

Afterwards, it was also determined the number of the bacterial (CFU/ mL) that internalized Caco-2 cells. After 1 h of Caco-2 cells/bacteria co-cultures, Caco-2 cells culture were incubated for 1 h with imipenem (IPM) in order to kill extracellular bacteria (attached cells), leaving viable only intracellular internalized cells (Figure 8.1).

When it is determined only the number of cells that internalized the cell of the Caco-2 cells line, can be found that the strain with a significantly higher internalized capacity was *P. fluorescens* AS008A1 (5.0 CFU/ mL, on average). Hence, when comparing the total number of microorganisms (attached and internalized) from this strain with the number of bacteria internalized into Caco-2 cells of each strain under study, it was noticed matching (no significantly different) results, which demonstrated that the all 6 strains assessed in the present study are featured by high internalization capacity. Anyway, the strain *S. putrefaciens* AS006C2 continues to be the strain that gives the smallest number of cells capable of internalization (4.39 CFU/ mL, on average).

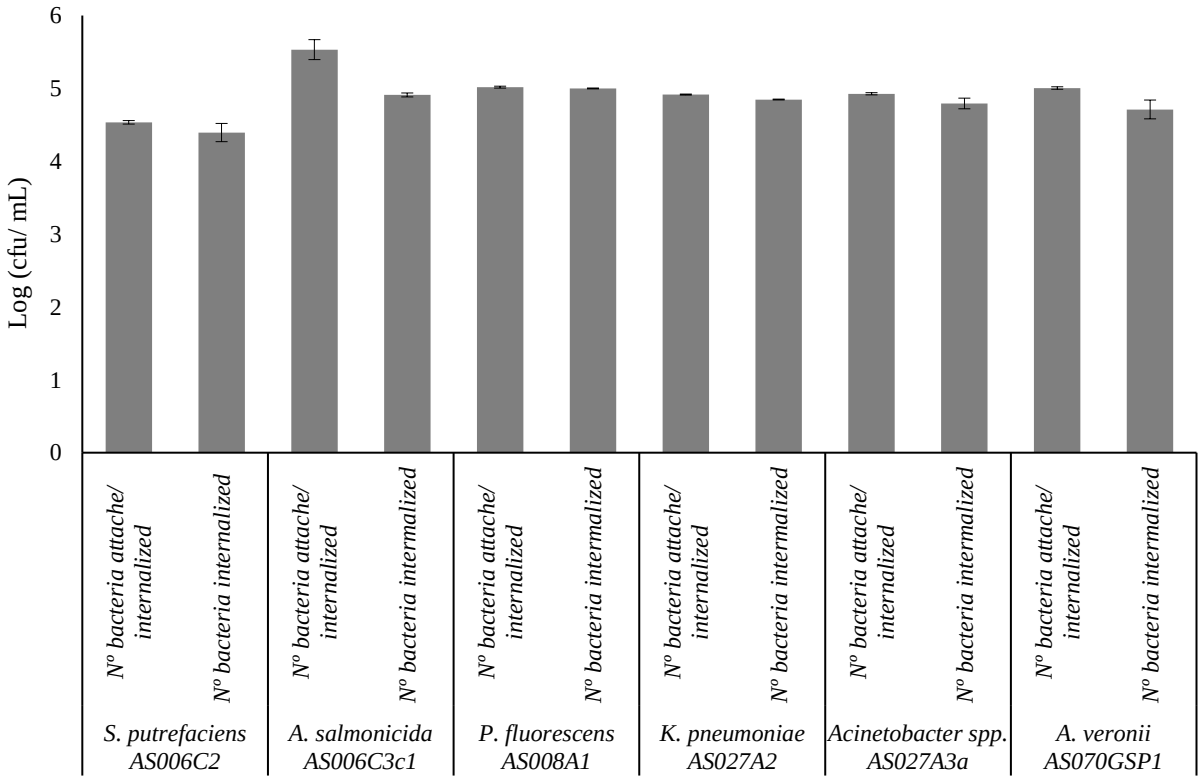

Figure 8.1 Log CFU/ mL of bacteria attached and internalized, and Log CFU/ mL of bacteria that internalized the human colon adenocarcinoma cell line Caco-2. Each value shown is the mean ± SD from three independent experiments.

The strains *A. salmonicida* AS006C3c1 (Figure 8.2) and *A. veronii* AS070GSP1 were the strains exhibiting the largest differences between the number of CFU/ mL of attached and internalized cells and the number of CFU/ mL of only internalized into Caco-2 cells.

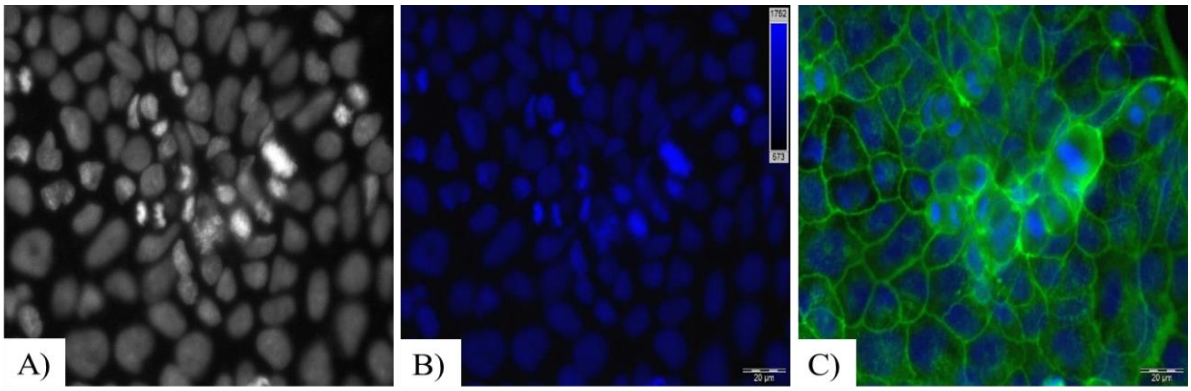

Figure 8.2 Human Caco-2 monolayers infected by *A. salmonicida* AS006C3c1 analyzed by conventional fluorescence microscopy, A) nuclear cells, B) fluorescence dye labelled nuclear cells, C) merged image.

8.4 DISCUSSION

The rapid emergence of resistant bacteria is occurring worldwide, jeopardizing the efficacy of antibiotics, which have historically contributed to lower the morbidity and mortality of a plethora of infectious diseases, saving millions of lives. However, nowadays, many years after the first patients obtained the benefits of antibiotic treatments, bacterial infections have again become a threat. Indeed, in the last decade, the antibiotic resistance has been due to their overuse and misuse, as well as a lack of developing new drug replacing the existing ones as microbial resistances were identified, mainly due to challenging regulatory requirements (Sengupta *et al.*, 2013). Hence, the augment of human activities and industrial processes have favored the development of this antibiotic resistance, as well as its dissemination in the environment, being conditioned by a varied range of biochemical and physiological mechanisms, which modulate the microorganisms virulence (Finley *et al.*, 2013).

In this work, it was studied the capacity of six multiresistant strains isolated from wild animals (*S. putrefaciens* AS006C2, *P. fluorescens* AS008A1, *K. pneumoniae* AS027A2, *Acinetobacter* spp. AS0027A3a, *A. salmonicida* AS006C3c1, *A. veronii* AS070GSP1) was to produce *N*-acyl homoserine lactone (AHLs) (C4-HSL; C6-HSL) was assessed. AHLs are signal molecules in quorum-sensing (QS) - regulated control of gene expression. *C. violaceum* CVO26, a Gram-negative microorganisms that can produce the purple pigment

through QS mechanism using AHLs signal molecules (Borges *et al.*, 2014), was used as bioindicator of AHLs activity test. Besides, it was tested the capacity of the strains to produce gelatinases, proteases, and siderophores, the roles of these enzymes in virulence have yet to be determined.

The determination of the capacity of six strains to adhere and invade human colon adenocarcinoma cells, by using Caco-2 cells as a differentiated intestinal epithelial cells from human colon intestine allowed to evaluate the number of attached and internalized bacterial cells (CFU/ mL). This cell line exhibits the morphological and functional characteristics of normal small intestinal cells (Kauffman *et al.*, 2013). Indeed, Caco-2 cell line is accepted as a substitute for human intestinal epithelial cells and are used to study the adhesion and invasion of bacterial pathogens (Nishikawa *et al.*, 1994).

In this study, it was stated that all six study strains showed the ability of attached and internalized into Caco-2 cells. The fact that *A. salmonicida* was the strain displaying the highest number of attached and internalized bacterial cells is in agreement with the recent review developed on the genus *Aeromonas* by Janda et al. (2010) reported that gastroenteritis is the most common infectious diseases associated to this strain. Actually, this genus has been gaining importance due to its increasing occurrence in human medicine (Jandaand Abbott, 2010). Structural factors have been described in *Aeromonas* strains that promote their attachment and colonization factors that protect the strains from host immune response (Tomas, 2012). Although *A. veronii* is one of the strains most frequently associated with human gastrointestinal diseases (Skwor *et al.*, 2014), in this study *A. veronii* $_{\text{AS70GSP1}}$ presented a smaller number of CFU/ mL attached and internalized than *A. salmonicida* $_{\text{AS006C3c1}}$. Furthermore, it appeared the strains of *Aeromonas* genus that showed a small capacity of invasion for Caco-2 cells. Several studies have demonstrated the adherence ability of *Aeromonas* spp. species to Caco-2 cell line (Rocha-De-Souza *et al.*, 2001, Guimarães *et al.*, 2002, Couto *et al.*, 2007, Santos *et al.*, 2011). In this sense, Nishikawa et al. (1994) also reported that *Aeromonas* strains can exhibit ability to invade Caco-2 cells.

Toxins production and adhesion ability are the most studied virulence factors in bacteria and have been shown to be the major determinant of pathogenicity for several multiresistant pathogenic bacteria (Ribetand Cossart, 2015). The internalization in host cells by bacteria is another strong factor associated with the pathogenicity of microorganisms, which may give access to environment protected from the host immune response and enable the survival and multiplication of bacteria (Kim *et al.*, 2010).

S. putrefaciens AS006C2 was the strain that has the smaller capacity to internalized Caco-2 cells line and is the only strains that is not able to produce AHLs.

The data retrieved upon this work also showed that the *P. fluorescens*AS008A1 was the strain that exhibiting the highest capacity to internalize human intestinal cells. Although *P. fluorescens* species has been considered as a psycrofile organisms, recent research has demonstrated that some strains of *P. fluorescens* might be able to grow at temperatures of 37 ºC or above (Chapalain *et al.*, 2008). These species are widespread in the environment, but studies showed that they can also be originate as a low level commensal of the human gastrointestinal tract (Wei *et al.*, 2002). The *P. fluorescens* AS008A1 strain used in this work also have the ability to produce gelatinases, proteases and AHLs. Several works have demonstrated that quorum sensing systems based on AHL signaling molecules are present in several *P. fluorescens* strains (El-Sayed *et al.*, 2001, Khan *et al.*, 2005, Liu *et al.*, 2007). Several extracellular virulence factors were found to be regulated by QS. This regulated expression of virulence factors is thought to give the strains a selective advantage over host defenses and thus is important for the pathogenesis of the bacteria (Smithand Iglewski, 2003). Extracellular proteins, such as gelatinases and proteases are recognized as virulence factors that cause diseases in fish and humans (Sudheesh *et al.*, 2012). The proteases promote development of the bacteria within the infected host and interfere with the host immune system (Hoge *et al.*, 2010)

Regarding *Acinetobacter* spp. AS027A3a and *K. pneumoniae* AS027A2, two important nosocomial pathogens, it was verified their similarity in Caco-2 cells attachment and internalization. In addition, both strains are not capable to produce proteases.

Using a universal chemical assay (CAS agar) for siderophores production, it was found that only *K. pneumoniae* AS027A2 produced siderophores. *K. pneumoniae* requires secretion of siderophores for bacterial replication and full virulence (Holden *et al.*, 2016). Siderophores are small, high-affinity iron-chelating molecules secreted by a wide variety of microorganisms that are critical for virulence in many Gram-negative bacteria (Holdenand Bachman, 2015). It is recognized that most *K. pneumoniae* infectious diseases are proceeded by colonization of intestinal epithelium (Di Martino *et al.*, 1997).

8.5 CONCLUSION

In conclusion, the data of this study reinforce the relevance of boosting the monitoring of the adverse impact of the environmental bacteria in human health, specially concerning those featured by resistance to antibiotic, as well as to understand that resistant microorganisms disseminate from animals to humans and vice versa, often through various environmental pathways. Thus, multiresistant strains isolated from wild animals can demonstrate ability to invade the human cells and thus cause disease. That is why a uniform and global surveillance strategy that complements existing strategies and includes analytical methods that can be used globally is needed to monitor the magnitude and dissemination of multi-resistant bacteria.

CHAPTER 9. CONCLUDING REMARKS AND PERSPECTIVES FOR FURTHER RESEARCH

9.1 MAIN CONCLUSION OF THIS WORK

Since the discovery and introduction of antibiotics, bacterial resistance has progressively increased. Humans have significant influence on the emergence of multiresistant bacterial populations in natural environments, due to the selective pressure exerted through waste management or agricultural practices. Wild animals, mainly species that live in close association with man, may be exposed to multiresistant bacteria in their environment, and antimicrobial resistance has been detected in fecal bacteria from a variety of wild animals, including mammals, reptiles, birds, and fish, worldwide.

Dissemination of antibiotic resistant bacteria and antibiotic resistance genes into the environment may also impact human health by contributing to the decrease of antibiotic therapy effectiveness.

The study here presented is a contribution to the knowledge on the presence of antimicrobial resistant bacteria recovered from different wild animals (mammals, reptiles and birds). The strain´s resistance phenotype was correlated to the presence of resistant genes determinants (*bla* genes) their virulence potential, their ability to adhered to a surface and form biofilms, their capacity to colonize the human gastrointestinal tract and invade human cells.

The main-conclusion that emerges from this work is that wild animals carry human associated antibiotic resistant bacteria and can be considered important reservoirs of antibiotic resistant determinants. Important multiresistant human pathogens, such as *Acinetobacter* spp., *K. pneumoniae* and *S. putrefaciens*, were observed in wild animal samples. Additionally, the presence of *Aeromonas* spp., emerging opportunistic human pathogens, responsible for a variety of infectious diseases in both immunocompetent and immunocompromised persons, was also detected.

Importantly the occurrence of bacterial strains exhibiting multiresistance profile and the presence of genes encoding *β*-lactamases has been demonstrated, including strains resistant to imipenem, an important intravenous *β*-lactam antibiotic. Because imipenem is used sparingly in clinical settings, being reserved for the most extreme cases in which other antibiotics are ineffective, one would expect most environmental strains to be sensitive. The β-lactamases genes were found in different combinations, and in one case, five β-lactamase genes were detected in the same strain, *A. veronii*. Eleven encoding sequences of OXA-aer, nine FOX and seven CphA, two MOX and one CTX-M genes were found and no TEM-, SHV-

, IMP- or VIM-types were identified. Increasing trends in bacterial multiresistance dictate the continued surveillance of bacterial communities associated with different environmental reservoirs.

Besides the observed antibiotic resistance phenotype, all the strains of this work showed ability to form biofilms (24, 48 and 72 h), which serve to protect bacteria from antibiotics and the host immune system, increasing their potential to cause persistent infections. Therefore, in order to identify potential therapeutic targets and strategies, it is important to understand biofilm development and the components of the protective biofilm matrix. Initial adhesion and biofilm formation on abiotic surfaces are important virulence mechanisms of bacteria to facilitate survival within host cells and in adverse environmental conditions. Our related studies suggest that these characteristics are strain-dependent and do not represent a species-specific phenotype or correspond to the source of bacterial strains, as all strains showed different abilities to adhered and form biofilm. Although strain-to-strain differences were observed in the individual infection models, overall, strains of environmental origin were found to be virulent, formation of biofilm indicative of disease progression and long-term persistence.

The results also suggest that all the strains presented some virulent potential, since all were able to produce gelatinase, and only the strain *S. putrefaciens* AS006C2 was not able to produce *N*-acyl homoserine lactones. *N*-acyl homoserine lactones are class of important signaling molecules involved in bacterial quorum sensing.

All strains were able to attach and to be internalized human colon adenocarcinoma Caco-2 cells line, indicating interaction of bacteria with gastrointestinal cell surfaces, which is believed to be a crucial first step in the colonization and subsequent infection of a host. Adhesion and invasion studies using human cell lines revealed a pronounced variation in the ability of the different strains to adhere to and invade epithelial cells. The fact that two strains *A. salmonicida* AS006C3c1 and *K. pneumoniae* AS027A2 were able to survive under human gastrointestinal conditions, points out to strains of environmental origin were as effective to colonizers human gastrointestinal tract and caused infection. Understanding the interaction of multiresistant bacteria with gastrointestinal human cells is a key to the assessment of their potential risks to human health, once gastrointestinal tract is believed to be the most important reservoir for transmission of the bacteria.

As a final conclusion, the results of this work suggest that it may be important to understand and monitory the impact of wildlife bacteria in public health, and elucidate how resistant bacteria disseminate from environment to man and vice versa, often through various environmental pathways.

9.2 FUTURE WORK

Some questions were raised by this study concerning bacterial communities present in sources for wildlife.

Concerning antibiotic resistance, the genetic determinants encountered not always explained the phenotypes expressed. On the other hand, the genetic bases of resistance to some antibiotics were not accounted for in this work.

The profiling of plasmid composition, dissection of their genetic content and potential for dissemination is an exciting and imperative field for investigation among our strains.

More details about virulence factors that are present in our strains as well as potential features of pathogenicity are required. It would be of paramount importance to establish a correspondence between the presence of the virulence factors identified and the putative pathogenicity of strains.

Additional assay of adhesion, invasion and cytotoxicity abilities of bacteria may be determined by the use of other different animal tissue cell lines.

Taken into account the importance of the biofilms formation in increasing antibiotic resistance, it would be necessary to performed more assay for the characterization of simple and multi species biofilms, and use more realistic models.

It would also be important established the genetic relationship among s of the same species collected from different animals and human. According to each situation, different experimental procedures described can be used: Pulsed Field Gel Electrophoresis (PFGE); and Multi-Locus Sequence Typing (MLST).

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