UNIVERSITÀ DEGLI STUDI DI NAPOLI
"FEDERICO II"

PhD Thesis

"The human-animal-ecosystem interface as reservoir of antimicrobial resistant microbes: the epidemiological role of wildlife and non-traditional animal species from a One Health perspective"

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C’è una virtù che molto amo, l’unica.

Essa ha nome tenacia.

Delle molte virtù di cui leggiamo nei libri
e di cui sentiamo parlare i maestri
non so che farmene.

E, d’altro canto,
tutte le molte virtù che l’uomo si è inventato
potrebbero essere raccolte
sotto un’unica denominazione.

Virtù significa obbedienza.

Solo che c’è da chiedersi a chi si obbedisce.

Anche la tenacia, infatti, è obbedienza.

Ma tutte le altre virtù,
tanto amate e lodate,
sono obbedienza a leggi
che sono state imposte da uomini;
soltanto la tenacia
non si inchina a queste leggi.

Chi è tenace
obbedisce infatti a un’altra legge,
una legge particolare,
assolutamente sacra,
la legge che ha in se stesso,
il “tenere a se stesso”.

(Hermann Hesse, Il coraggio di ogni giorno)

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Grazie di cuore a tutti,

Lorena

ABA	Anaerobe basal agar
ACE	Angiotensin Converting Enzyme
AgNPs	Silver Nanoparticels
AIPDZ	Associazione Italiana Petauri dello Zucchero
AMPs	Antimicrobial Peptides
AMR	Antimicrobial resistance
ARB	Antimicrobial Resistant Bacteria
ARDB	Antibiotic Resistance Database
ARGs	Antimicrobial Resistant Genes
BLAs	β-lactam antibiotics
BLIs	β-lactamase inhibitors
BPA	Baird-Parker agar
BPW	Buffered-Pepton Water
CARD	Comprehensive Antibiotic Resistance Database
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CAZ-AVI	Ceftazidime-Avibactam
CBA	Columbia blood agar base
CBFA	*Campylobacter* blood-free selective agar
CCDA	*Campylobacter* blood-free selective agar
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
CIN	Cefsulodin-Irgasan-Novobiocin agar
CLSI	Clinical Laboratory Standards Institute
CMM	Cooked meat medium
CRE	Carbapenem-resistant Enterobacteriaceae
CRISPR	Clustered Regularly Interspaced Short Palindromic
CSEB	Campylobacter-selective enrichment broth
CTX	(Cefotaximase)-M type beta-lactamases
DABCOs	Diazabicyclooctanes
DBOs	Diazabicyclooctanones
DSFs	Diffusible Signal Factors
ECDC	European Center for Disease Control and Prevention
EFSA	European Food Safety Authority
EPS	Extracellular Polysaccharides
ESBL	Extended Spectrum β-Lactamase
FAO	Food and Agriculture organization
GAP	Global Action Plan
GML	Glycerol Monolaurate
HGT	Horizontal Gene Transfer
HI	*Hermetia illucens*
HIL	Hermetia illucens *larvae*
IMI-REL	Imipenem/Relebactam
IMP-Class B	Imipenemase metallo-β-lactamase
KPC-Class A	*K. pneumoniae* carbapenemase
LA	Lauric Acid
MCA	MacConkey agar
MCFAs	Medium-chain Fatty Acids
MCRs	Melanocortin Receptors
MDK	Minimum Duration of Killing
MDR	Multidrug resistant bacteria
MEM-VAB	Meropenem/Vaborbactam

List of abbreviations

MIC	Minimum Inhibitory Concentration
MLST	Multi-locus sequence typing
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSH	α-Melanocyte-Stimulating Hormone
MTSB	Modified Tryptone Soy Broth
NAP	Nactional Action Plan
NCS	Coagulase-Negative Staphylococci
NDM-Class B	New Delhi metallo-β-lactamase
OCLA	Chromogenic *Listeria* agar
OIE	Office International des Epizooties
OXA	(Oxacillinase)-type beta-lactamases
OXA-48-Class D	Oxacillinase-48
PBP	Penicillin binding protein
PBS	Phosphate-buffered saline
PCA	Pseudomonas cetrimide agar
PFGE	Pulsed-Field Gel Electrophoresis
PMQR	Plasmid-mediated quinolone resistance
PUFA	Poly-unsaturated Fatty Acids
QS	Quorum Sensing
RV	Rappaport-Vassiliadis Broth
SCFAs	Short-chain Fatty Acids
SHV	(Sulfhydryl variable)-type beta-lactamases
SMCA	Sorbitol MacConkey agar
SNPs	Single Nucleotide Polymorphisms
STEC	Shiga toxin-producing *E. coli*
STs	Sequence Types
SVL	Snout-Vent Length
TEM	(Temoneira)-type beta-lactamases
UTIs	Urinary Tract Infections
UVM I	*Listeria* primary selective enrichment medium
UVM II	*Listeria* secondary selective enrichment medium
VIM-Class B	Verona integron-encoded metallo-β-lactamase
VRE	Vancomycin resistant *Enterococcus faecium*
VRSA	Vancomycin resistant *S. aureus*
WHO	World Health Organization
WWTP	Wastewater treatment plant
XLD	Xylose Lysine Desoxycholate agar
ZnSO4	Zinc Sulfate

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Abstract

Antimicrobial resistance (AMR) is a global public health threat that affects human, animal and ecosystem health. The antibiotic resistance crisis has been attributed to the worldwide overuse and misuse of antimicrobials in both human and veterinary medicine, as well as the lack of new drug development by the pharmaceutical industry due to reduced economic investment and challenging regulatory requirements. The global spread of antibiotic resistance genes (ARGs) and their acquisition by clinically relevant microorganisms mainly occur through horizontal gene transfer of mobile genetic elements, genetic mutation and recombination, and the proliferation of antimicrobial resistant bacteria due to selective pressures that are imposed by antimicrobial compounds or other contaminants, such as biocides or heavy metals. Antibiotic resistance hot spots are found not only in medical settings but also in environmental compartments that are subjected to anthropogenic pressure and contribute to the reservoir of ARGs collectively constituting the antibiotic resistome. Basing on a holistic and multidisciplinary "One Health" approach to face the AMR, scientific research highlighted the need to reveal the missed link between the increasing of AMR in livestock and humans and the emergence of AMR in wildlife. Since wildlife species exist across multiple trophic levels occupying a particular niche and interact with the environment in different and specific ways, they are suitable candidates to accumulate and spread resistance determinants acting as sentinels for the ecosystem health. Due to their feeding and migratory behavior, wild birds are considered the main reservoir and long-distance disperser of both commensal and pathogenic bacteria and, therefore, they have often been used as model species to determine the antimicrobial resistance profiles of key indicator pathogens (e.g., the Gram-positive methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant Enterococci and Extended-spectrum β-lactamases producing Enterobacteriaceae) and to delineate the evolution of AMR. Although some progress has been made in understanding the epidemiology of multi-host infections involving wildlife, limited data are available on the role of wildlife in the spread of antibacterial resistance. This PhD thesis was undertaken with the aim to give a broader consideration to this research topic since the discussion of AMR is often focused on human and livestock health outcomes. The studies reported have been performed to assess the antimicrobial resistance profiles of bacteria from wild species (wild birds, reptiles), as well as in other non-traditional species that in recent years have become popular as pets. The detection of multidrug-resistant strains of clinically relevant pathogens in wild and/or captive species of reptiles, birds and small mammals from different settings suggests that they may act as potential carriers of zoonotic agents and resistance determinants, highlighting the potential risks for public health related to the close contact and the sharing of the same environment. Although these results are preliminary and need further investigations to build knowledge in the field, we emphasize the need to address this topic from a "One Health" perspective, since human, animal and ecosystem health are interdependent and require an integrated approach to contain the global threat of AMR. The "One Health" concept has been adopted in 2015 with the launch of the first global action plan (GAP) by the WHO, OIE and FAO, in order to develop solutions and measures to control health risks attributable to AMR, identifying surveillance, infection prevention, responsible use, universal access and research development as the main policy goals to tackle AMR. In this context, the discovery and development of new antimicrobials represent a global priority, however in the last decades only a few new antibiotics/antibiotic combinations have been approved for clinical use. To date, numerous natural and synthetic alternatives to conventional antibiotics have been investigated, some of which have progressed while others still are in their nascent stage and further research is needed to assess the efficacy and exploit the potential of these new strategies. With this in mind, this thesis ends with a focus on an insect-derived medium-chain fatty acid, the lauric acid and its monglyceride derivative, glycerol monolaurate, which exhibit a broad spectrum of microbicidal activities. The high content of this fatty acid within insects, as well as their numerous bioactive components which provide many additional benefits to animal health, highlight the potential of insect lipids as antimicrobials and make them favorable feed and food options from the perspective of animal and human nutrition in the current global scenario where a sustainable and circular economy is required.

I. The global threat of Antimicrobial Resistance in the current scenario

In 1928, with the discovery of penicillin by Sir Alexander Fleming, the modern era of antibiotics started and transformed modern medicine saving millions of lives. However, as early as 1945, Fleming raised the alarm regarding antibiotic overuse giving warning that the "public will demand [the drug and] … then will begin an era … of abuses." (Fleming, 1945). The antibiotic resistance crisis has been attributed to the worldwide overuse and misuse of antimicrobials, as well as the lack of new drug development by the pharmaceutical industry due to reduced economic investment and challenging regulatory requirements. Thus, many decades after the miracle of antibiotics, rapidly emerging resistant bacteria threaten the extraordinary health benefits that have been achieved with antibiotics requirements (Ventola, 2015). In the last decade, the US Centers for Disease Control and Prevention (CDC), the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO) are considering infections caused by multidrug resistant (MDR) bacteria as an emergent global disease and a major public health problem (WHO, 2014; CDC, 2019). Antimicrobial resistance (AMR) increases health-care costs, length of stay in hospitals, morbidity and mortality in both developed and developing countries. A recent report estimated that 10 million deaths will be attributed to AMR by 2050 (Founou et al., 2017). Despite warnings regarding overuse, antibiotics are overprescribed or inappropriately prescribed worldwide in both human and veterinary medicine. Large amounts of antibiotics have been used as growth promoters as well as for prophylaxis, methaphylaxis and the treatment of infections among farm animals and in aquaculture, raising the selective pressure on both commensal and pathogenic microbes that can spread to humans through direct contact and via the food chain or indirectly from the environmental pollution of farm effluents (Roca et al., 2015). This abuse has also caused the accumulation of these compounds in the environment, turning it into an enormous reservoir for antibiotic resistance genes.

The WHO published the first global surveillance report on AMR in 2014 to show the clinical impact of resistant bacteria in WHO regions across the world. This report highlighted that five out of the six WHO regions had more than 50% resistance to third generation cephalosporins and fluoroquinolones in *Escherichia coli* and methicillin resistance in *Staphylococcus aureus* (MRSA) in hospital settings. Similarly, more than 50% resistance to third generation cephalosporins and carbapenems was reported in *Klebsiella pneumoniae*. The report further revealed that *K. pneumonia*e resistant to third generation cephalosporins was associated with elevated deaths in Africa (77%), the Eastern Mediterranean region (50%), South East Asia (81%) and Western Pacific region (72%) (Zhen et al., 2019). Among gram-positive pathogens, a global pandemic of resistant *S. aureus* and *Enterococcus* species currently poses the biggest threat. The emergence of MDR (and increasingly pan-resistant) gram-negative bacilli has affected practice in every field of medicine. The most serious gram-negative infections occur in health care settings and are most commonly caused by Enterobacteriaceae (mostly *Klebsiella pneumoniae*), *Pseudomonas aeruginosa*, and *Acinetobacter* spp. MDR gram-negative pathogens also encompass extended-spectrum beta-lactamase-producing *Escherichia coli* and *Neisseria gonorrhoeae* (Ventola, 2015). The term "ESKAPE" includes six such pathogens with growing multidrug resistance and virulence: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. ESKAPE pathogens are responsible for majority of nosocomial infections and are capable of "escaping" the biocidal action of antimicrobial agents. They recently gained further global attention by being listed by the WHO as priority antibiotic-resistant bacteria (WHO, 2017), according to the urgency of need for new antibiotics. Carbapenem resistant *A. baumannii* and *P. aeruginosa* along with extended spectrum β-lactamase (ESBL) or carbapenem resistant *K. pneumoniae* and *Enterobacter* spp. are listed in the critical priority list of pathogens, whereas vancomycin resistant *E. faecium* (VRE) and methicillin and vancomycin resistant *S. aureus* (MRSA and VRSA) are in the list of high priority group (Tacconelli et al., 2018).

Table 1. WHO priority pathogens list for research and development of new antibiotics.

Priority 1: Critical	Priority 2: High	Priority 3: Medium
Acinetobacter baumannii, Carbapenem-resistant	*Enterococcus faecium*, Vancomycin-resistant	*Streptococcus pneumoniae*, Penicillin non susceptible
Pseudomonas aeruginosa, Carbapenem-resistant	*Staphylococcus aureus*, Methicillin-resistant, Vancomycin intermediate and resistant	*Haemophilus influenzae*, Ampicillin-resistant
Enterobacteriaceae, Carbapenem-resistant Third-generation Cephalosporine-resistant	*Helicobacter pylori*, Clarithromycin-resistant	*Shigella* spp., Fluoroquinolone-resistant
	Campylobacter, Fluoroquinolone-resistant	
	Salmonella spp., Fluoroquinolone-resistant	
	Neisseria gonhorreae, Third-generation Cephalosporine-resistant, Fluoroquinolone-resistant	

II. Mechanisms of resistance acquisition

The mechanisms of multidrug resistance exhibited by ESKAPE are broadly grouped into three categories namely, drug inactivation commonly by an irreversible cleavage catalyzed by an enzyme, modification of the target site where the antibiotic may bind, reduced accumulation of drug either due to reduced permeability or by increased efflux of the drug. The first resistance strategy involves enzymatic inactivation of the drug, by either degrading or modifying the antimicrobial compound such that it no longer has activity. Examples of this mechanism include β-lactamases, which degrade β-lactam antimicrobials, tetracycline destructases, such as Tet(X), and chloramphenicol acetyltransferases. The second resistance strategy is overexpression, modification, or protection of the drug target in the bacterium, enabling survival. This mechanism is used by MRSA, which expresses PBP2a, a redundant and methicillin-insensitive version of the native PBP2 penicillin-binding protein encoded by the *mecA* gene. ESKAPE pathogens are also able to form biofilms that physically prevent the immune response cells of host as well as antibiotics to inhibit the pathogen (Adu-Oppong et al., 2017; Mulani et al., 2019). In some cases, super-resistant strains have also acquired increased virulence and enhanced transmissibility. Realistically, antibiotic resistance can be considered a virulence factor. Phenotypic analyses of partial or "complete" gene knockout libraries by saturation mutagenesis of bacterial genomes permit the identification of specific mutants eliciting hypersensitivity responses to antibiotics. It is assumed that overexpression of the corresponding wild-type gene would generate a resistance phenotype (Davies and Davies, 2010).

Antibiotic resistance exhibited by bacteria can be intrinsic, acquired, or adaptive. The development of resistance to antibiotics can occur naturally (intrinsic) based on a spontaneous gene mutation in the absence of selective pressure from antibiotics. Examples of intrinsic resistance include the glycopeptide resistance exhibited by Gram-negative bacteria due to the impermeability of the outer membrane present in the Gram-negative bacterial cell envelope (Christaki et al., 2020). Bacteria

develop resistance to antibiotics (acquired) when at least one bacterium within a heterogeneous bacterial colony acquires a resistance mechanism by either a mutation or the acquisition of new genetic material from an exogenous source. The acquisition of foreign genes or mobile genetic elements by organisms or between species, is referred to a horizontal gene transfer (HGT), which may occur through three main mechanisms: transformation, transduction and conjugation (Fletcher, 2015). Adaptive resistance is defined as the resistance to one or more antibiotics induced by a specific environmental signal (e.g., stress, growth state, pH, concentrations of ions, nutrient conditions, sub-inhibitory levels of antibiotics). Subinhibitory and subtherapeutic antibiotic concentrations can promote the development of antibiotic resistance by supporting genetic alterations, such as changes in gene expression, HGT and mutagenesis. Changes in antibiotic-induced gene expression can increase virulence, while increased mutagenesis and HGT promote antibiotic resistance and spread. Low levels of antibiotics have been shown to contribute to strain diversification in organisms such as *Pseudomonas aeruginosa*. Subinhibitory concentrations of piperacillin and/or tazobactam have also been shown to induce broad proteomic alterations in *Bacteroides fragilis* (Ventola, 2015). Since integrons and other mobile genetic elements allow bacteria to adapt faster to new niches, genes mobilized in the future would likely not be restricted to conferring antibiotic resistance but may also encompass genes that provide a fitness advantage in terms of adaption to changing environments. Thus, genes allowing bacteria to survive highly variable abiotic conditions, handle toxicants, utilize novel carbon sources, compete with other microbes, adhere to different types of surfaces, and allow formation of highly durable spores would be good candidates for future mobilization. This paints a picture of a bleak future in which human pathogens would not only be non-treatable by most antibiotics, but also would become more aggressive and spread more easily between humans (Bengtsson-Palme et al., 2018).

Apart from resistance, bacteria can survive antibiotics action through another mechanism, the tolerance. A tolerant population exhibits no difference in the minimum inhibitory concentration (MIC) compared to a susceptible population and cannot replicate during treatment, but it can survive high doses of bactericidal antibiotics (Sulaiman and Lam, 2021). Tolerance can be quantified by measuring the minimum duration of killing (MDK) of the population, namely the time it takes to reduce the population by a certain percentage at a certain dose of antibiotic (Fridman et al., 2014). The tolerant subpopulation, called persisters, are present naturally in almost every bacterial populations. Several mechanisms and pathways have been implicated in the phenotypic switch to a persister state, such as the stringent response and (p)ppGpp signaling, RpoS and the general stress response, SOS response, bacterial communication and quorum sensing, and Toxin/Antitoxin modules (Harms et al., 2016). Although persister cells do not differ genetically from their susceptible counterparts in the same population, it has been shown that the "level of persistence" can be modulated by genetic changes. Tolerance mutations may be caused by the antibiotic treatment itself or occur spontaneously, with tolerant mutants remaining undetected in the population and being able to take it over during the course of repetitive antibiotic treatments. To give more insights into the bacteria's adaptation mechanisms, it would be expected to map by "omics" methodologies the "tolerome", namely the complex of genes and proteins in which mutations affect the tolerance level of the cells (Sulaiman and Lam, 2021).

The rapid detection of resistance mechanisms plays an important epidemiological role in surveillance studies to evaluate the potential dissemination of resistance genes. Molecular methods have shortened the time to detect specific resistance mechanisms and the development of next generation sequencing technologies has increased the number of sequenced bacterial genomes at an exponential rate (Roca et al., 2015). The ability to sequence DNA from environmental and clinical samples (metagenomics and whole genome sequencing) to determine gene content provides the opportunity to identify antimicrobial resistant genes (ARGs) as well as understand the mechanism behind their acquisition. ARGs that all convey a particular resistance can be compared to determine if they were derived from vertical inheritance by one or more groups or acquired horizontally by taxonomically unrelated groups. When the genome sequence of an organism is available and taxonomy is known, it is possible

to determine if an ARG was gained through HGT by comparing the phylogenetic relationship of two distinct organisms, to the homology of their ARGs. Access to complete genome sequences facilitates the ability to distinguish between ARGs located on a plasmid (a common source of HGT) and ARGs or single nucleotide polymorphisms (SNPs) that are incorporated into an organism's genome (Brinkac et al., 2017).

III. Antimicrobial resistance hot spots

Despite the widespread and growing evolution of AMR, limited focus has been placed on the role of environmental factors in propagating resistance. Acquisition of resistance genes by bacterial communities may or may not occur in response to selective pressure. The presence of antibiotics in the environment exerts pressure which induces stress response from the microbial community promoting HGT and the sharing of resistance genes as a bacterial adaptation response (Fletcher, 2015). Antibiotic resistance hot spots are found not only in medical settings but also in environmental compartments that are subjected to anthropogenic pressure and contribute to the reservoir of antimicrobial resistance genes (ARGs) collectively forming the antibiotic resistome (Berendonk et al., 2015). The term "resistome" has been proposed to describe the ecology of resistance genes, broadly encompassing all resistance genes circulating in pathogenic and non-pathogenic bacteria, be they from soil, animals, humans, or other sources (Hoelzer et al., 2017). For the transfer of resistance to human pathogens, the abundance of pathogenic bacteria that can act as recipients is crucial. This means that the human microbiome could potentially play a role in this process, and that human commensals may act as intermediary resistance reservoirs. The human microbiome is a complex ecosystem consisting of trillions of microbes closely interacting and exposed to resistance determinants. Rates of HGT among bacteria in the human microbiome (primarily the human GI microbiome) have been estimated to be about 25 times higher than among bacteria in other diverse microecosystems like soil (Bengtsson-Palme et al., 2018). Studies suggest that 20% to more than 30% of the human gut microbiota may exhibit multidrug resistance in the absence of little to no antibiotic exposure, with quinolone resistant alleles existing at notably high frequencies within host-associated bacteria (Berendonk et al., 2015). Furthermore, the presence of intrinsic resistance genes in the environment (e.g., genes present in manure, sewage, hospital waste) significantly contributes to environmental pollution with resistance genes. Reports have shown that antibiotic resistance genes which are currently present in human pathogens, have been detected in pristine environments and in humans and animal populations that have never been in contact with antibiotics. This suggests that antibiotic resistance genes which have integrated in successful gene-transmission elements could persist and spread in the environment even in the absence of antibiotics (Fletcher, 2015).

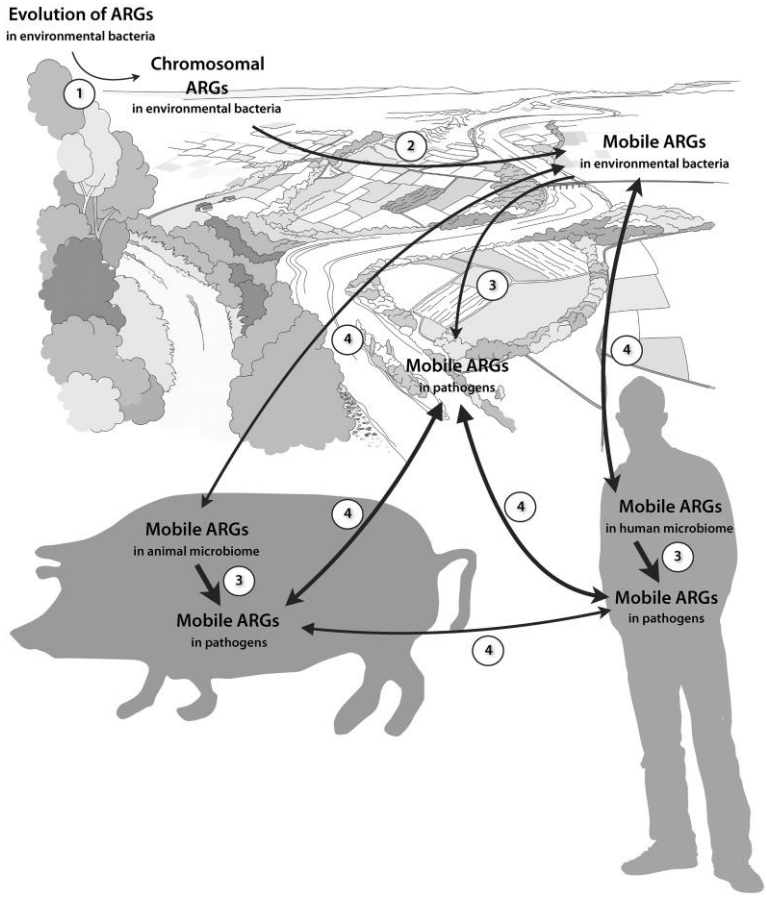

Fig. 1. The circular transfer of ARGs in the human-animal-environment interface (Bengtsson-Palme et al., 2018).

Numerous types of anthropogenic activity, including antibiotic use in agriculture and aquaculture, other nonhuman applications of antibiotics and waste disposal also affect the environmental microbiome (Davies and Davies, 2010). Besides a large number of different genetic mechanisms that facilitate gene transfer, the modern globalized world has provided efficient ways for bacteria and resistance genes to rapidly disseminate worldwide through trade of food products or live animals, and people travelling. Animal-to-human transmission can easily occur through multiple routes, of which the direct transmission via fresh food products, such as meat and eggs, probably is the most important. The transfer of resistant bacteria to humans by farm animals was first noted more than 35 years ago. An estimated 80% of antibiotics sold in the U.S. are used in animals, primarily to promote growth and to prevent infection (Ventola, 2015). Antimicrobial-resistant bacteria may also transfer from animals to humans through direct contact. This is, for example, the case for MRSA CC398, where transmission frequently occurs from the animals to the farmers or other people in contact with the animals (Aarestrup, 2015). Up to 90% of the antibiotics given to livestock are excreted in urine and stool, thus soil receives a large portion of excreted antibiotics through application of manure and sewage sludge as fertilizers. Soil inevitably becomes a hot spot for antibiotics to affect indigenous microbes (Fletcher, 2015).

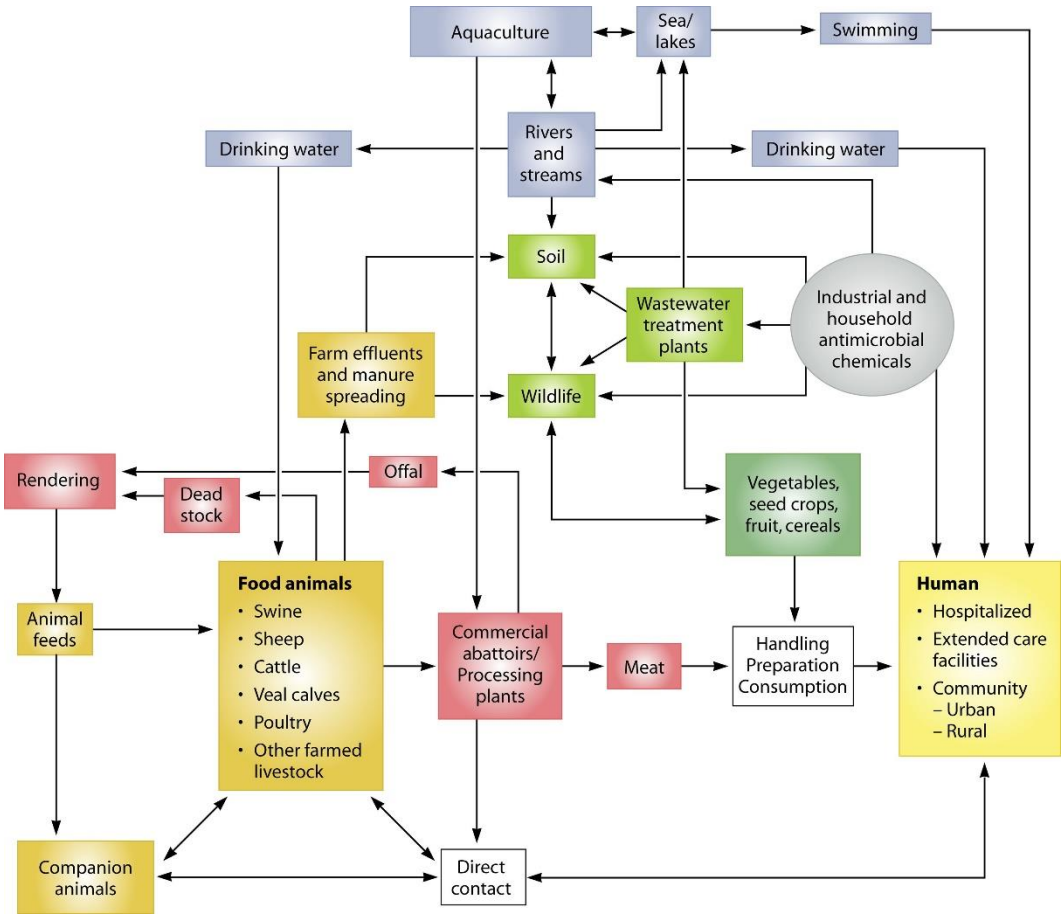

Fig. 2. The interlinked factors that contribute to perpetuate the AMR cycle (Davies and Davies, 2010).

The transfer of resistance genes to pathogens from environmental bacteria, which occupy other habitats and are often less phylogenetically related, would likely be less common, although environmental stressors may induce horizontal gene transfer to and from (opportunistic) human pathogens in environmental settings. This means that once a resistance factor has entered a human pathogen, it is more likely to further spread between commensals and pathogens than being transferred again into another pathogen from environmental bacteria, and in any case the consequences/contribution from the latter type of event would be expected to be small. Finally, transfer of resistance factors from human pathogens to environmental bacteria is possible, enabling human-associated bacteria to use environmental bacterial populations as reservoirs for resistance genes that can later be re-recruited into the human-associated resistome. Wild birds and animals living close to humans are also known to harbour bacteria carrying resistance genes and may contribute to spreading those genes across large areas. Once a resistance gene is widely spread among human pathogens (or even among human commensals), we are restricted to manage its spread. Mitigation of the spread of resistance factors to human pathogens should therefore ideally take place before they get a foothold in the human microbiome. Thus, detection of resistance determinants in the environment that are not yet widespread among clinical bacteria should be an important component in risk assessment and management of antibiotic resistance (Bengtsson-Palme et al., 2018).

IV. Concluding remarks and needs for research

Since antimicrobial resistance is a complex and multifaceted problem affecting humans, animals and the environment, detecting and controlling it requires a holistic and integrated ‘‘One Health’’ approach (Karp et al., 2017). The ‘One Health’ approach was interpreted more broadly to encompass collaboration not only across human and animal sectors but also economic, social and behavioral components with a focus on collaborative action in this area. The new strategy had a major focus on measuring outcomes alongside an innovative combination of policy approaches to stimulate research and development, improve surveillance, prevent and control infections, optimize antimicrobial use and relay key AMR messages to public and professional audiences (Shallcross et al., 2015). To combat this emerging global threat in a comprehensive manner, recent international efforts have resulted in the adoption of a Global Action Plan (GAP) in 2015 by the WHO as the main policy goal to tackle AMR, emphasizing the interdependence of human health, animal health, and the environment (WHO, 2015; Hoque et al., 2020; Ogyu et al., 2020).

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Objectives

The overall aim of this thesis was to give a broader consideration to the role of wildlife and other non-conventional animal species as drivers of AMR in the human-animal-ecosystem interface, since the discussion of AMR is often focused on human and livestock health outcomes.

With this in mind, the first chapter of the thesis provides an overview of the significant literature concerning temporal and geographical trends, epidemiological aspects and molecular characterization of resistant bacteria detected in wildlife. To build further knowledge on this topic, the following chapters are intended to assess the antimicrobial resistance profiles of bacteria from wild species (wild birds, reptiles, mammals), as well as other non-traditional species become popular as pets in the last years. Basing on the "One Health" concept, these studies have been performed to highlight the interconnection between humans, animals and environment in perpetuating the AMR cycle, the implications for public health and the need of further research directly linking anthropogenic sources of specific resistance patterns in wildlife populations.

Finally, the present assessments and future perspectives of the integrated approach to tackle the multifaceted and complex challenge of AMR will be summarized, particularly with respect to the development of novel therapeutic strategies. In this regard, the last chapter provides a perspective on the potential of the insect-derived medium-chain fatty acid, the lauric acid, as a promising and sustainable alternative to antibiotics in the current AMR scenario.

Chapter 1

Antimicrobial Resistance in wildlife and other non-conventional animal species: an overview of the significant literature

1.1 Introduction

While the increasing prevalence of AMR in both clinically relevant and commensal bacteria in livestock and humans can be attributed largely to selection through the use of antimicrobials, interest in environment, including wildlife, has gradually gained attention in order to better understand the effects of anthropogenically-derived antimicrobial pollution and resistance in ecosystems (Swift et al., 2019). Through a holistic and multidisciplinary "One Health" approach to face the AMR, many authors emphasized the need to reveal the missed link between the increasing of AMR in livestock and humans and the emergence of AMR in wildlife. Publications related to this research topic dated from 1979 to 2019 and, overall, the temporal trend in publication on antimicrobial resistance in wildlife shows a growth in the number of studies published per year, with 78% of the research papers published in the last ten years (Torres et al., 2021).

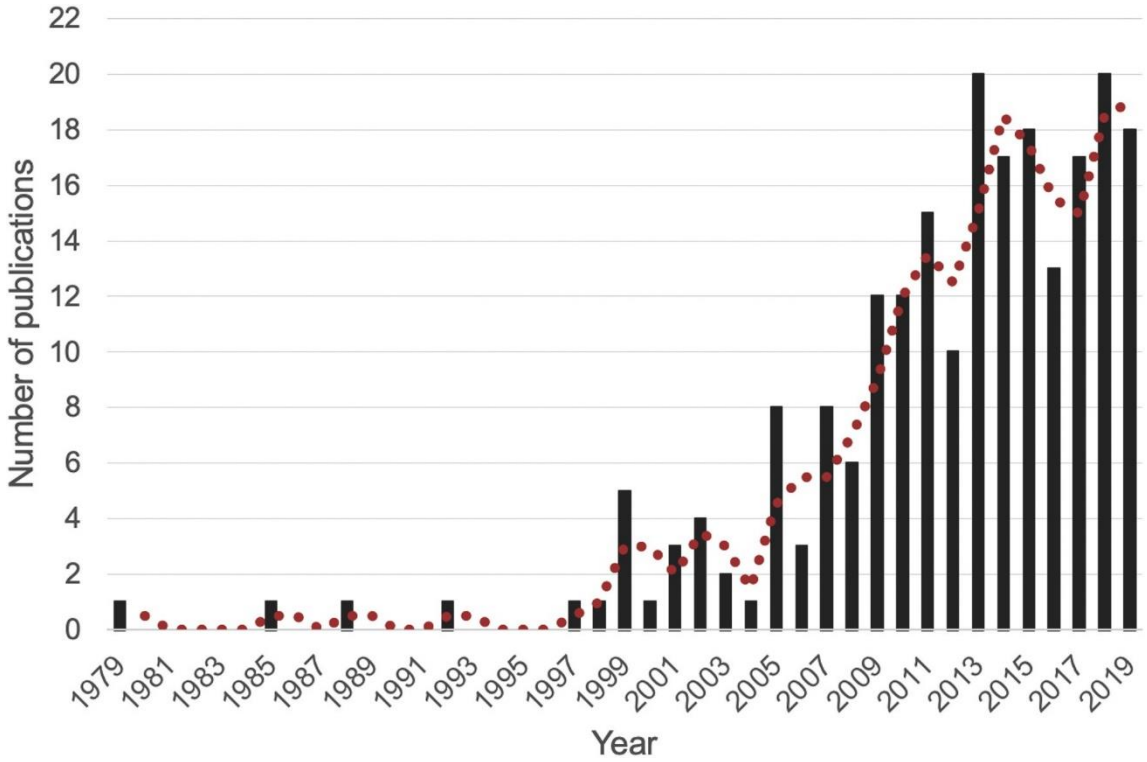

Fig. 1.1 The increasing of published papers on AMR in wildlife from 1979 to 2019 (Torres et al., 2021).

The origins of antimicrobial resistance in the wildlife are important to human health both for the growing threat of new zoonotic diseases, of which about 70% arise from wildlife reservoirs, and the need for predicting emerging resistant pathogens (Radhouani et al., 2014; Torres et al., 2021). In 1977, antimicrobial resistant *E. coli* isolates originating from wildlife species were reported for the first time in Japanese wild birds and 5 years later in South African baboons feeding on human refuse (Guenther et al., 2011; Guyomard-Rabenirina et al., 2020). Since the first report of ESBL- producing *E. coli* from wild animals in Portugal in 2006, at least 80 wildlife species worldwide have been reported to carry ESBL-producing Enterobacteriaceae, including mostly waterfowl, birds of prey and rats, but also in species living in environments that are less exposed to human activities (Wang et al., 2017). For example, resistance to ciprofloxacin, a relatively recently developed and completely synthetic antimicrobial, was detected even in the most remote groups of monkeys in Mexico (Arnold et al., 2016).

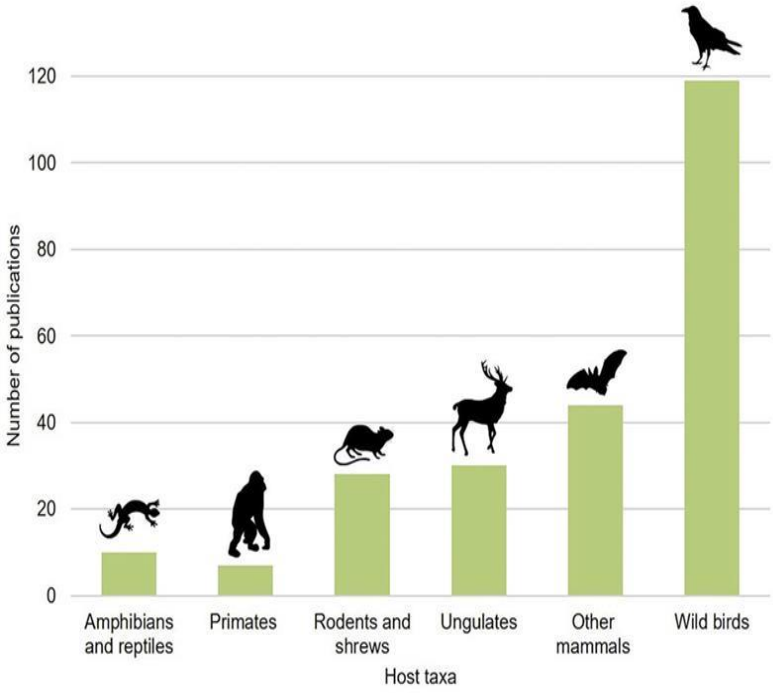

Fig. 1.2. Number of papers related to host taxon group (Torres et al., 2021).

Even within wildlife populations living in close contact with humans or livestock and their wastes, there is little evidence directly linking an anthropogenic source of AMR with specific patterns of resistance and/or resistance genes, thus the role of wildlife might be underestimated. (Swift et al., 2019). Additionally, more information is also needed to understand if wild animals are only temporary carriers of bacteria with plasmid-mediated resistance or if these bacteria are able to persist in their gut (Dolejska and Papagiannitsis, 2018).

Wildlife exists across multiple trophic levels and is therefore a suitable candidate to accumulate and spread resistance determinants within ecosystems. Ecological features, such as habitat, feeding and ranging behavior could define the exposure of wildlife to antimicrobial resistance, and how widely it is dispersed in the environment. As land use changes reduce the availability of natural habitats, wildlife species are forced to look for alternative sources of food and shelter, bringing them into closer contact with humans, livestock and their waste, and increasing the potential for transfer of antimicrobial resistance between them (Hassel et al., 2019). Although wild animals are not treated with antibiotics, their ability to easily move across environmental gradients of humanization (from pristine – natural – agroforestry – to highly humanized scenarios) can enhance their contact with commensals and/or resistant bacteria promoting adaptation mechanisms and horizontal transfer of resistance genes within the bacterial community of wildlife (Torres et al., 2021).

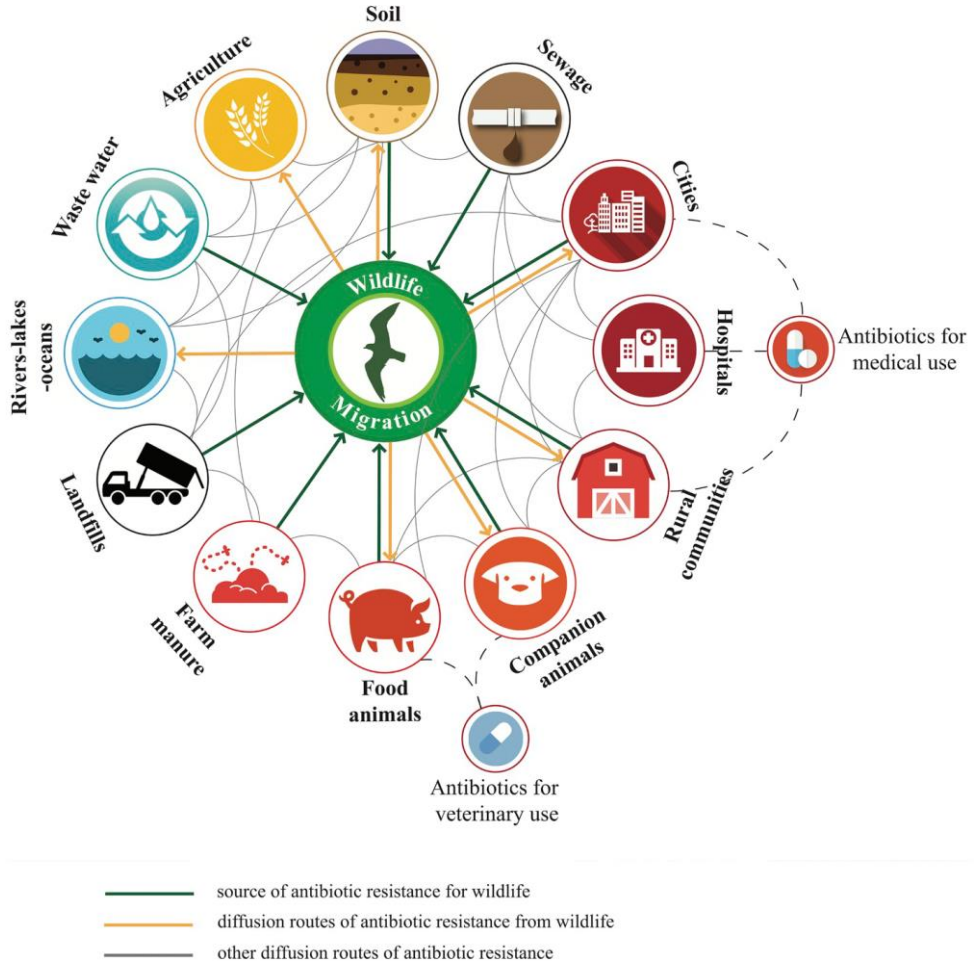

Fig. 1.3. Transmission pathways of antibiotic resistance between wildlife, livestock and humans (Dolejska and Papagiannitsis, 2018).

The consequences for wildlife of the evolution of AMR in commensal, or even pathogenic bacteria are still untested but probably neglectable, while their role as remarkable source of AMR for humans or livestock represents the major issue. To control the transmission of wildlife infections to humans and livestock it is suggested to follow three main approaches: separation of, or reducing contact with the wildlife source, vaccination and wildlife population control, often by culling. Vaccination is not possible for AMR control, and the physical separation of wildlife from livestock is difficult and often unworkable. The protection of the human food chain from AMR is crucial but challenging due to the importance of wild game, seafood and bushmeat both nutritionally and culturally in many human communities (Arnold et al., 2016).

In conclusion, wildlife communities provide a unique opportunity to investigate landscape dynamics of antimicrobial resistance, as each species occupies a particular niche and interacts with the environment in different and specific ways, acting as sentinels for ecosystem health (Jobbins and Alexander, 2015). Although some progress has been made in understanding the epidemiology of multi-host infections involving wildlife, less attention has been paid to the role of wild animals in the ecology and evolution of AMR. Therefore, the following sections of the chapter will provide an overview on the detection of resistant bacteria and related ARGs in the main wildlife species involved in the evolution and dispersion of AMR within environments. Similarly, the AMR topic will also be analyzed with regard to other non-conventional animal species such as reptiles and companion birds. Since these species are becoming popular as pets worldwide, we would put the focus on their potential as carriers of zoonotic and resistant bacteria to address some missing points regarding the epidemiology and the implications for public health in light of the “One Health” concept.

1.2 Key indicator pathogens to trace the evolution of multi- resistant bacteria in the environment and wildlife

Al large number of bacteria are involved in the phenomenon of multi-resistance and nosocomial infections in both human and veterinary medicine, while as far as the environment and wildlife are concerned, it is recognized that the Gram-positive MRSA, VRE and ESBL producing Gram-negative bacteria like *Escherichia coli* are the main key indicator pathogens to delineate the evolution of AMR (Guenther et al., 2011; Radhouani et al., 2014). *E. coli* is ubiquitous and colonize the gut of birds and mammals both as commensal and/or pathogen that requires the use of antibiotics. It is also estimated that *E. coli* spends approximately half its life cycle in the external environment, and therefore, environments (soil, surface or ground water) contaminated with these antibiotic-resistant organisms and genes may constitute a reservoir for their dissemination (Carroll et al., 2015).
The occurrence of ESBL-*E. coli* in the environment dated two decades after the first outbreaks in human clinical settings (Costa et al., 2006) and in wildlife shortly after its first detection in livestock farming, probably due to the horizontal transfer of resistance genes from clinical isolates or the intake of already resistant bacteria from human waste, sewage, and domesticated animal manure (Kummerer, 2009). Most studies on ESBL-*E. coli* in wildlife originate from Central Europe, an area with high livestock and human density and an assumable frequent interaction of wildlife with human influenced habitats like livestock farms, landfills, sewage systems or wastewater treatment facilities, resulting in a higher risk for wildlife acquiring antibiotic resistant bacteria (Guenther et al.,2011). On the other hand, several studies reported the occurrence of ESBL-*E. coli* in remote places suggesting an influence of migratory behavior of wild birds into remote areas or the ubiquitous presence of human influence in various ecological niches of the planet mainly via human feces (Hernandez et al.,2010).

1.2.1 Extended-spectrum β-lactamases (ESBL) producing Enterobacteriaceae

The term extended-spectrum β-lactamases defines the ability to hydrolyze a broad spectrum of beta-lactam antimicrobials containing an oxyimino-group such as oxyimino-cephalosporins (e.g., ceftazidime, cefotaxime) as well as oxyimino-monobactam (aztreonam), whereas they are not active against cephamycins and carbapenems. They are usually inhibited by beta-lactamase-inhibitors like clavulanic acid and tazobactam, which determines a difference between ESBL and AmpC–beta-lactamases producing bacteria. ESBLs have been found in a wide range of Gram-negative bacteria, but mostly belonging to the family of Enterobacteriaceae, such as *Klebsiella* spp., *E. coli*, *Salmonella enterica*, *Citrobacter* spp. and *Enterobacter* spp. (Bradford, 2001). The four enzyme families currently referring to the most common ESBLs among Enterobacteriaceae are: TEM (Temoneira)-type beta-lactamases, SHV (Sulfhydryl variable)-type beta-lactamases, CTX (cefotaximase)-M type beta-lactamases, and OXA (oxacillinase)-type beta-lactamases (Livermore, 1995; Pfeifer et al., 2010; Naseer and Sundsfjord, 2011). In parallel to the current situation in human and veterinary medicine, the type of extended-spectrum beta-lactamases found in wild animals are clearly dominated by the *bla*CTX-M gene-family, while other genes are surely less prevalent. In order to trace the evolution of AMR in ESBL-*E. coli*, besides comparative whole genome analysis, the multi-locus sequence typing (MLST) might be a very useful approach. Based on a small set of marker genes, MLST analysis revealed the existence of strains belonging to identical sequence types (STs) and being isolated from different hosts, showing a common phylogeny and therefore a zoonotic potential for most strains analyzed so far (Wirth et al., 2006). Indeed, same clusters of *E. coli* causing systemic infections in birds, and urinary tract infection and neonatal meningitis in humans have been reported, suggesting the possible scenarios of transmission through different routes from humans to wild birds and vice versa (Guenther et al., 2010). The pandemic spread of certain ESBL-*E. coli* strains (e.g., CTX-M-15-producing ST131 *E. coli*) (Wang et al., 2017) poses a serious challenge in human clinics and underlies the complexity of dissemination of antimicrobial drug resistance, therefore further spatial and

temporal studies are needed to fully determine the role of wildlife as source and carrier of antimicrobial resistance determinants.

1.2.2 Carbapenemase producing Enterobacteriaceae (CPE)

Carbapenems often represent the last line of defense in the treatment of infections with multi-resistant Gram-negative pathogens, however the emergence in the last years of carbapenem resistance has threatened the clinical utility of this antibiotic class worldwide. Carbapenem-resistant bacteria can arise from β-lactam ring hydrolysis by dedicated carbapenemase enzymes or from changes in membrane permeability via mutations in efflux pumps or porins coupled with ESBL expression. This group of beta-lactamases is very diverse and can be found in three different β-lactamase classes, such as Ambler Class A or D serine β-lactamases and Ambler class B metallo-β-lactamases (Potter et al., 2016). *K. pneumoniae* carbapenemase (KPC-Class A), New Delhi metallo-β-lactamase (NDM-Class B), Verona integron-encoded metallo-β-lactamase (VIM-Class B), Imipenemase metallo-β-lactamase (IMP-Class B) and Oxacillinase-48 (OXA-48-Class D) variants are the most common carbapenemases in CPE, although several emerging enzymes are gradually being described (Meletis, 2016). The high occurrence of carbapenemase genes on MDR plasmids transferred both within Enterobacteriaceae and other bacterial families is considered a major global health concern (Harmer and Hall, 2015). In Europe, carbapenem-resistant Enterobacteriaceae (CRE) are emerging causes of human infections in many countries resulting in an endemic situation in 2015. Epidemiological studies indicated intensive care therapy, hospital admission and previous travel outside the country of residence as major risk factors for acquiring CRE among humans (Grundmann et al., 2017). In veterinary medicine, even if the use of carbapenems is not allowed for livestock, the occurrence of CRE in German swine farms in 2012, as well as in companion animals has been reported (Kock et al., 2018). CRE in wildlife have been reported in Europe, Africa, Asia and Australia, with CRE isolates harbouring blaOXA-48 in wild boars and wild storks, blaIMP, blaVIM-1 and blaKPC-2 in silver and yellow-legged gulls, and blaNDM-1 in a black kite (Bachiri et al., 2017; Bouaziz et al., 2017; Dolejska et al., 2016; Vittecoq et al., 2017; Fischer et al., 2013; Vergara et al., 2017). These findings demonstrated that these carbapenemases are prevalent in the environment leading to colonization of wildlife which may act as vectors in the spread of CRE beyond borders of farms, dwelling zones, countries, or even continents. Therefore, prevention of CRE occurrence and spread in wildlife, food-producing and companion animals should be a major public health priority to protect both persons with direct exposure and consumers.

1.2.3 Vancomycin-resistant Enterococci (VRE)

Enterococci are ubiquitous microorganisms of the human and animal gut microbiota, but they are also recognized as nosocomial opportunistic pathogens with increased resistance to antimicrobial approved agents. The significant increasing prevalence of VRE inside and outside the hospital environment is considered a global health problem. The emergence of VRE with acquired mechanisms of resistance, especially those harbouring *vanA* or *vanB* genes, has been reported worldwide in humans and animals with different frequencies (Ben Yahia et al., 2018). Incidence of VRE among wild animals has been described in several countries (Mallon et al., 2002; Figueiredo et al., 2009; Ishihara et al., 2013; Lozano et al., 2015; Klibi et al., 2015; Stepien-Pysniak et al., 2018), including in remote areas of the world (Silva et al., 2011). The detection rates of acquired VRE in wild birds from Europe, America, Africa and Australia ranged between 1.3% and 4% (Ben Yahia et al., 2018; Oravcova et al., 2017; Silva et al., 2012; Roberts et al., 2016). The *vanA* gene has been reported both in *E. faecium* and in *E. fecalis* isolated from wild birds (Santos et al., 2013; Oravcova et al., 2016; Ben Yahia et al., 2018; Klibi et al., 2018). The increasing detection of VRE in wildlife with acquired and clinically relevant mechanisms of resistance should be evaluated in terms of public health implications, and its evolution should be analyzed and monitored in the future using new high

throughput technologies to completely understand the evolution of predominant clones in different hosts and environments.

1.3 Antibiotic-resistant bacteria: a host species perspective

The available scientific literature has shown the detection of antimicrobial resistance, especially among commensal gut bacteria, in wild mammals, birds, reptiles and fish, with prevalence and resistance patterns varying across species, geographic area and possibly time (Sousa et al., 2011; Carroll et al., 2015; Swift et al., 2019; Garcês et al., 2019; Guyomard-Rabenirina et al., 2020). It is also recognized that the highest rates of AMR observed in wildlife are related to the degree of association with human activity (Jobbins and Alexander, 2015), however several studies reported the occurrence of MDR bacteria in remote areas of the world (Hernandez et al., 2010). Due to their feeding and migratory behavior, wild birds are considered the main hosts for antimicrobial resistance bacteria within wildlife, with waterfowl and birds of prey being the predominant species involved in this phenomenon. Fecal pollution of water by human or livestock sources may explain the AMR transmission scenario in waterfowl, while birds of prey, being on top of the food chain, could accumulate resistant bacteria and related determinants from their typical prey, like mice and shrews, that are synantropic species often in close contact with humans and livestock. (Kozak et al., 2009).
In the following sections of the chapter will be summarized and discussed the main findings of the current literature regarding the wildlife host spectrum of AMR, particularly with respect to wild birds, companion birds and reptiles.

1.3.1 Wild birds

Previous and recent literature suggested that wild birds could be a reservoir and long-distance disperser of clinically relevant microorganisms, thus they have been used as model species to determine antimicrobial resistance profiles (Table 1.1). Due to their diversity in ecological niches and also in the composition of the gut microbiota within the different avian species, data on the carriage of MDR bacteria are heterogeneous and reflect the complexity of the spread of AMR through environments.
Among wild birds, pigeons and gulls have a close proximity to human activities that led to modify their diet habits and become omnivorous and opportunistic, feeding mainly on trash, landfills and occasional supply provided by some residents (Morakchi et al., 2017). Pigeons colonize cities and proliferate largely in the absence of predators. In addition, parks and beaches represent a meeting place for domestic and human animals, urban birds and environmental waters. Thus, this close contact promotes the possible horizontal transfer of bacteria between humans and pigeons. Human pathogenic bacteria carrying multidrug resistance determinants may reach the pigeons' gut via food, and humans can be infected by contaminated pigeon droppings (da Silva et al., 2012; Morakchi et al., 2017). As peri-domestic wildlife, pigeons and gulls are recognized as carriers in the global transmission of ESBL, AmpC β-lactamase, carbapenemase and colistin resistance genes in Enterobacteriaceae (Bonnedahl et al., 2009; Dolejská et al., 2009; Migura-Garcia et al., 2017; Morè et al., 2017; Ngaiganam et al., 2019). In particular, there have been several publications presenting data from various gull species in the last years, although the majority were restricted to the geographical area or in the time span of the sampling. Interestingly, to cover this gap, Stedt et al. (2014), investigated the spatial variation of *E. coli* resistance levels in a cross-sectional survey in gulls across Europe, evaluating the dissemination of resistant strains in the environment. The results showed considerable variation between countries, with Iberian countries being in the upper range of *E. coli* resistant levels, while Ireland and Denmark had a smaller proportion of resistant isolates. The diversity of MDR phenotypes was substantial, with 59 different resistance combinations detected, of which more than one clone per country was involved. The most frequently recovered phenotypes were resistant to tetracycline or ampicillin, which represent the most commonly antibiotics used in both veterinary and human medicine for decades, while other resistance phenotypes such as mecillinam and nalidixic acid emerged surprisingly, with nalidixic acid resistant levels resulting even higher than the levels of human clinical isolates in Spain. Other studies have reported that gulls frequently carry resistant *E.*

coli with human and/or livestock-associated genotypes, however, due to their migratory behavior, it is difficult to correlate spatially and temporally all these data. Furthermore, to assess the potential of gulls introducing resistant bacteria to new areas far from the origin, it should be clarified for how long they shed these bacteria and how long they persist in the environment. Interestingly, in 2015 Masarikova et al., documented the presence of identical clusters of resistant *Salmonella* strains from a wastewater treatment plant (WWTP) and nestlings of black-headed gulls (*Chroicocephalus ridibundus*) in Czech Republic. Based on the wide ranges of serotypes detected, the authors hypothesized that the *Salmonella* isolates from the WWTP were of both human and animal origin and that the colonization of young gulls was linked to the spread from the WWTP to the water environment and then via the feed collected by their parents directly. Moreover, since the first discovery in domestic swine in China, gulls have also been reported as source of environmental contamination by colistin-resistant bacteria harbouring the mobilized colistin resistance gene (*mcr-1*) (Liakopoulos et al., 2016; Ruzauskas et al., 2016; Ahlstrom et al., 2019; Franklin et al., 2020). Colistin is a polymyxin antibiotic of last resort used in treating multi-drug resistant bacterial infections in humans. The horizontal transfer of this mobile genetic element among MDR bacteria (e.g., carbapenem-resistant Enterobacteriaceae) represents a serious concern for human health, making some bacterial infections difficult or impossible to treat. In their experimental trial, Franklin et al. (2020) provided evidence that ring-billed gulls (*Larus delawarensis*) may be readily colonized with at least one strain of *mcr-1* positive *E. coli*, exhibit shedding patterns indicative of bridge hosts, shed bacteria in feces for an extended period (e.g., 16 days), which can be detected in the environment for even longer (e.g., 29 days), and are able to infect conspecifics occupying shared environments. These important findings confirmed that gulls have the potential to disseminate clinically important colistin resistance through environmental pathways.

As mentioned above, a wide range of wild birds are recognized as important reservoirs and vectors of resistant bacterial pathogens, as well as bioindicators of AMR in the environment, such as waterbirds, corvids, starlings, birds of prey and passerines (Guenther et al., 2010; Carroll et al., 2015; Giacopello et al., 2016; Söderlund et al., 2019), as shown in Table 1.1. The feeding and behavioral ecology of each species may explain their ecological role in the transmission of pathogenic and resistant microorganisms within the human-livestock-environment interface.

Table 1.1 Presence of ESBL-producing E. coli in wild birds.

Reference	Animal species	Country	ESBL Type	MLST
Costa et al. (2006)	Bird of prey Owl	Portugal	*bla*TEM-52 *bla*TEM-52 +*bla*CTX-M-14 *bla*CTX-M-14 +*bla*TEM-1 *bla*CTX-M-1 +*bla*TEM-1, *bla*SHV-12 *bla*CTX-M-14	
Poeta et al. (2008)	Seagulls (Larus sp.)	Portugal	*bla*TEM-52, *bla*CTX-M-1 *bla*CTX-M-14, *bla*CTX-M-32	
Dolejska et al. (2009)	Black headed-gull (*C. ridibundus*)	Czech Republic	*bla*CTX-M-1, *bla*CTX-M-15, *bla*SHV-2, *bla*SHV-12	
Bonnedahl et al. (2009)	Yellow legged gull (*L. michahellis*)	France	*bla*CTX-M-1, *bla*CTX-M-1 +*bla*TEM-1 *bla*CTX-M-15 +*bla*TEM-1, *bla*TEM- *bla*SHV +*bla*TEM-1	ST1199, ST533, ST1140, ST156, ST90, ST1142, ST681, ST1134, ST1143 ST1135, ST1144, ST746 ST351
Hernandez et el. (2010)	Glaucous winged gull (*L. glaucescens*)	Russia	*bla*CTX-M-14, *bla*CTX-M-15	ST131, ST609, ST746
Bonnedahl et al. (2010)	Black headed gull (*C. ridibundus*)	Sweden	*bla*CTX-M-14, *bla*CTX-M-15	ST1646, ST1340, ST1647
Literak et al. (2010)	Mallard duck (*A..platyrhynchos*) Herring gull (*L. argentatus*)	Poland	*bla*CTX-M-1, *bla*CTX-M-9 +*bla*TEM-1b *bla*CTX-M-15 +*bla*OXA-1, *bla*SHV-12	
Pinto et al. (2010)	Buzzard (*B. buteo*) Barn owl (*T. alba*) Tawny owl (*S. aluco*) Boot eagle (*A. pennata*) Montagu's harrier (*C. pygargus*), Black kite (*M. migrans*), Black vulture (*C. atratus*), Bonelli's eagle (*A. fasciata*), Eurasian eagle owl (*B.bubo*), Raven (*C. corax*)	Portugal	*bla*CTX-M-1, *bla*CTX-M-1 +*bla*TEM-1, *bla*CTX-M-1 +*bla*TEM-20, *bla*SHV-5, *bla*SHV-5 +*bla*TEM-1, *bla*SHV-5 +*bla*TEM-20	
Radhouani et al. (2010)	Buzzards (*B. buteo*)	Portugal	*bla*CTX-M-32 +*bla*TEM-1, *bla*CTX-M-1 +*bla*TEM-1	
Simoes et al. (2010)	Se*agu*lls (*Larus* sp.)	Portugal	*bla*CTX-M-1, *bla*CTX-M-9 *bla*CTX-M-15, *bla*CTX-M-32	ST1284, ST131, ST224, ST453, ST86, ST205, ST359, ST165, ST69, ST1152, ST405, ST559, ST1163, ST10, ST58, ST155, ST297, ST43, ST58, ST156
Guenther et al. (2010)	Eurasian blackbird (*T. merula*), White fronted goose (*A. albifrons*), Rock pigeon (*C. livia*)	Germany	*bla*CTX-M-15	ST648
Garmyn et al. (2011)	Wild geese (*B .canadensis*, *A. anser*)	Belgium	*bla*TEM-52, *bla*SHV-12	ST1079, ST1844
Guenther et al. (2012)	Black Kites (*Milvus migrans*) Red kites (*Milvus milvus)* Buzzard (*Buteo buteo*)	Germany	*bla*CTX-M-1	ST12, ST744, ST847, ST1640, ST2199, ST2198

Table 1.1 Presence of ESBL-producing E. coli in wild birds (continued).

Reference	Animal species	Country	ESBL Type	MLST
Tausova et al. (2012)	Great cormorants (*Phalacrocorax carbo*)	Czech Republic Slovak Republic	*bla*CTX-M-15, *bla*CTX-M-27	ST131
Loncaric et al. (2013)	Rooks (*Corvus frugilegus*)	Austria	*bla*CTX-M-1, *bla*CTX-M-3 *bla*CTX-M-15, *bla*TEM-15	ST23, ST34, ST58, ST69, ST90, ST131, ST162, ST491, ST744, ST1683
Bonnedahl et al. (2014)	Gulls	Alaska	*bla*CTX-M-14, *bla*CTX-M-27, *bla*CTX-M-15, *bla*TEM-19	ST10, ST38, ST131, ST405, ST2253, ST2967
Hasan et al. (2014)	Brown-headed gull (*C. brunnicephalus*)	Bengal	*bla*CTX-M-14, *bla*CTX-M-15	ST10, ST48, ST131, ST345, ST349, ST648, ST853, ST1727, ST2687, ST2688, ST2689
Bàez et al. (2015)	Franklin's gulls (*Leucophaeus pipixcan*)	Chile	*bla*CTX-M-15, *bla*CTX-M-2, *bla*CTX-M-22, *bla*CTX-M-3, *bla*TEM-40, *bla*TEM-198	ST10, ST38, ST44, ST131, ST205, ST350, ST359, ST405, ST540, ST617, ST642, ST665, ST744, ST3476, ST4184, ST4185, ST4186, ST4187, ST4188, ST4189
Hassan and Shobrak (2015)	Common kestrel (*Falco tinnunculus*)	Saudi Arabia	*bla*CTX-M	
Rashid et al. (2015)	Open bill stork (*Anastomus oscitans*)	Bangladesh	*bla*CTX-M-15	ST2689, ST4016
Alcalà et al. (2016)	White stork (*Ciconia ciconia*), Black kite (*Milvus migrans*), Red kite (*Milvus milvus*), Golden eagle (*Aquila chrysaetos*), Griffon vulture (*Gyps fulvus*)	Spain	*bla*CTX-M-1, *bla*CTX-M-14, *bla*SHV-12	ST10, ST38, ST57, ST131, ST155, ST156, ST453, ST744, ST877, ST1158, ST1431, ST3788
Liakopoulos et al. (2016)	Kelp gull (*Larus dominicanus*)	Argentina	*bla*CTX-M-14, *bla*CTX-M-2	ST101, ST744
Parker et al. (2016)	Crow	Canada	*bla*CTX-M-15, *bla*SHV-2	
Fuentes-Castillo et al. (2019)	Magellanic Horned-Owl (*Bubo magellanicus*), Rufous legged-Owl (*Strix rufipes*)	Chile	*bla*CTX-M-8	ST2705, ST345
Ngaiganam et al. (2019)	Yellow legged gulls Pigeon	France	*bla*CTX-M-1, *bla*CTX-M-15, *bla*TEM-1, *bla*CTX-M-15	ST10, ST38, ST857, ST34

1.3.2 Pet Birds

Nowadays, pet birds are the third most common companion animal after dogs and cats, with the majority of cage birds belonging to two orders: Passeriformes including canaries, finches, and Psittaciformes including parrots, parakeets, and lovebirds (Sigirci et al., 2020). Bacteria can be present on or within a bird's body for an extended period without causing overt clinical disease signs before the animal appears to suddenly exhibit signs of illness. Normal fecal bacteria cultured from healthy birds include Gram-positive bacilli (e.g., *Lactobacillus* spp., *Bacillus* spp., *Corynebacterium* spp., and *Streptomyces* spp.) and cocci (e.g., *Staphylococcus epidermidis*, *Streptococcus* spp., *Aerococcus* spp., and *Micrococcus* spp.). Gram-negative bacteria occasionally recovered in clinically normal birds include *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Citrobacter* spp., *Pasteurella* spp., *Moraxella* spp., and *Pseudomonas* spp. (Giacopello et al., 2015). The close contact between household pets and humans poses a hazard to human health because of their potential as source of both zoonotic pathogens and MDR bacteria via contaminated food, environment or direct contact. In addition, the administration of antibiotics in pet bird farms without medical control is a very common practice, contributing to the increasing resistance rates (Varriale et al., 2020). Although the public health implications related to this topic are not negligible, poor information on the antimicrobial resistance in these species is available in the literature. So far, only few studies investigated Gram-negative infections in Passeriformes and Psittaciformes with the related AMR profiles. The most commonly recovered resistant phenotypes are shown in Table 1.2.

Table 1.2 Percentages of E. coli resistant phenotypes in domestic Passeriformes and Psittaciformes.

Reference	Country	*E. coli* resistant phenotype
Giacopello et al., 2015	Italy	AMC (91.2%), TE (87.5%), DO (85%), OT (50%), SXT (46.2%)
Ylmaz et al., 2017	Turkey	*AMC (100%), AMP (100%), TE (100%), S (50%), SXT (50%)
Di Francesco et al., 2018	Italy	E (84.1%), SP (76.5%), AML (76.5%), CS (47.1%), TE (41.2%)
De Pontes et al., 2018	Brazil	AML (81%), AMP (78%), S (74%), TE (41%), ENR (30%)
Varriale et al., 2020	Italy	AMC (66.6%), OT (57.4%), ENR (55.5%), DO (48.1%), SXT (46.3%)
Sigirci et al., 2020	Turkey	**TE (84%), SXT (46%), S (34%), KA (25%), SAM (19%)

AMC: Amoxycillin/Clavulanic Acid; SXT: Trimethoprim/Sulfamethoxazole; DO: Doxycycline; ENR: Enrofloxacin; OT: Oxytetracycline; TE: Tetracycline; E: Erythromycine; SP: Spyramycin; AML: Amoxycillin: CS: Colistin Sulphate; S: Streptomycin; KA: Kanamycin; SAM: Ampicillin-Sulbactam.
* The percentages refer exclusively to the ESBL producing *E. coli*.
** The percentages encompass the overall Enterobacteriaceae resistant isolates, including *E. coli*.

In the above-mentioned studies the differences between the AMR rates could result from several factors, including geographical and host species differences, sampling techniques, and detection procedures. The few studies reported focused mostly on phenotypic resistance profiles, however Sigirci et al. (2020), Ylmaz et al. (2017) and De Pontes et al. (2018), conducted PCR analysis for screening some of the ARGs. Noteworthy, Sigirci et al. (2020), reported for the first time the presence

in a parrot and two parakeets of plasmid-mediated quinolone resistance (PMQR) in three MDR isolates harbouring *qnrB* and *qnrS* genes. In addition, they noticed of seven aminoglycoside-resistance genes (*aac*(3)-*IIa*(aacC2), *strA*, *strB*, *aadA*(*aadA1* or *aadA2*), *aphA1*, *aphA2*, and ant(*20'*)-*Ia*(*aadB*)) in three MDR isolates from parakeets. In their survey, Ylmaz et al. (2017) showed that two (50 %) of the four ESBL-producing *E. coli* isolates produced CTX-M-1 and the other two (50%) produced CTX-M-15. Regarding other β-lactamase, TEM-1b was produced by all ESBL-producing *E. coli* isolates. Through phylogenetic grouping, all ESBL positive isolates were classified into the B1 phylogenetic group, suggesting that ESBL genes were disseminated between commensal *E. coli* isolates in birds. Pulsed-Field Gel Electrophoresis (PFGE) revealed clonal similarity in CTX-M-producing *E. coli*, in accordance with the data reported by Guenther et al. (2011) in wild birds, assuming that same dominant ESBL types may spread in the environment with possible transmission between birds and/or birds and humans. Finally, De Pontes et al. (2018) detected resistant genes to aminoglycoside in 77% of the *E. coli* strains, in 54% to penicillin, in 35% to teracycline and sulphonamide and in 4% to quinolones, with *strAB* and *blaTEM* being the most frequently detected genes. Increased Gram-negative resistance is mainly attributed to mobile genetic elements such as plasmids, which may be disseminated within bacterial communities even across continents by air travels and animal transport. These findings provided important novel data to build knowledge in this field, highlighting the role of cage birds in the spread of AMR determinants and the potential risks for public health. In the scope of "One health", further studies and a multidisciplinary approach based on surveillance programs, appropriate use of antibiotics and collaboration between professionals and owners are highly recommended.

1.3.3 Reptiles

Wild and captive reptiles may be exposed to antimicrobial resistant bacteria directly or indirectly through different sources. In the recent years, exotic reptiles have risen in popularity as pets, resulting in a trade of non-conventional species around the world, with a population of over 7 million in European households (FEDIAF, 2019). The close contact between reptiles and their owners has favored the conditions for the transmission of zoonotic infections, especially regarding Salmonellosis, as these species are considered asymptomatic carriers of *Salmonella* spp. (Mughini-Gras et al., 2016). However, only about 6% of human salmonellosis cases are acquired after direct or indirect contact with reptiles, since contaminated food from animal origin represent the main transmission source (EFSA and ECDC, 2019). More recently, MDR *Salmonella* strains have emerged as a potential concern for public health, thus it has been included in the WHO priority list of antibiotic-resistant bacteria. In view of "One Health" concept, interest in the role of reptiles as an antibiotic-resistant *Salmonella* reservoir has increased, even if the different mechanisms by which wild and captive reptiles acquire and spread resistance determinants should be deepened. Interestingly, Marin et al. (2021) detected 48% of *Salmonella* strains in pet reptiles (lizards, snakes and chelonians) of Eastern Spain, of which 72% were MDR. Twenty-five different patterns of resistance were observed, with gentamicin-colistin (18.7%) and gentamicin-colistin-ampicillin (10.7%) as the most predominant. Gentamicin is an aminoglycoside used indiscriminately in the chelonian industry, especially in the US, the main supplier country of reptiles for the European market, as prophylactic *Salmonella* treatment in eggs (Diaz et al., 2006), and this may explain the high percentage of resistance recovered (84%) in this survey. Resistance to ampicillin was also reported (46.7%), as well as in other studies conducted on pet reptiles in Italy (18%) (Russo et al., 2018), in India (Singh et al., 2013), in Taiwan (Chen et al., 2010) and in California (Gorski et al., 2013). Due to direct or indirect contact between reptiles and their owners, transmission of resistance determinants is possible both from humans to reptiles and vice versa, and also within different bacterial populations.

Reptiles are not considered natural carriers of *E. coli* and few data on AMR are available. At our knowledge, Guyomard-Rabenirina et al. (2020) reported the first case of ESBL-producing *E. coli* in

a wild reptile (*Iguana iguana*) of Guadeloupe, although it was not closely related to the isolates that cause human disease in this region, suggesting that wild animals are not a direct source of infection for humans and that human invasive *E. coli* are not passed to wildlife.
Resistant bacteria reptile-associated have also been detected in the wild species, even in areas with low exposure to human activity (Dipineto et al., 2018; Abrahão et al., 2020; McWorther et al., 2021). Abrahão et al. investigated the prevalence and the AMR profiles of *Salmonella enterica* in invasive exotic lizards (*Salvator merianae*) from an inhabited Brazilian archipelago, showing a prevalence of 43.8% but a low antimicrobial resistance (13.3% to Colistin, 10.2% to Sulfamethoxazole, 1% to Ampicillin, Amoxicillin/Clavulanate, Nalidixic Acid and Ciprofloxacin). These findings suggest that the geographical isolation of the island population hampers contact with strains from livestock living in the continent, where antimicrobial resistance is common in *S. enterica*. Similarly, in 2018 Dipineto et al. evaluated the bacterial prevalence of an insular population of Italian lizards (*Podarcis sicula klemmeri*) with the related AMR profiles. No strain of *Salmonella* spp. was detected, *Citrobacter* spp. showed a multidrug resistance phenotype (Ampicillin- Amoxicillin/Clavulanate- Doxycycline-Streptomycin), whereas *Enterobacter* spp. and *E. coli* 0145 were resistant to ampicillin. It is difficult to compare and speculate regarding the results emerged from the above-mentioned studies on wild reptiles, various factors not yet fully understood may affect the frequency and variation in antimicrobial resistance, although the anthropogenic impact and feed are supposed to be key factors. However, local geographical pressures and complex mechanisms for the evolution of AMR within the environment are also likely to play an important role.

1.4 Conclusions and research gaps

This overview was aimed to give more attention to some aspects of the AMR topic and highlight the role of wildlife and other non-traditional animal species in the AMR cycle from a "One Health" perspective. AMR is a global threat that does not affect humans and animals separately but should be addressed by analyzing the complexity of mechanisms underlying the evolution and the ecology of AMR across environments. With this in mind, we emphasized the importance of wildlife in this scenario, since wild species, especially wild birds, are recognized as remarkable reservoirs and vectors of resistant bacteria, as well as bioindicators of environmental pollution. So far, scientific research has made great efforts in order to assess and quantify the contribution of wildlife in the spread of resistant determinants, mainly by genomics, that allowed to trace the epidemiology of AMR comparing the similarity of clones detected in wildlife, livestock and human clinical settings. On the other hand, by reviewing the literature many research gaps have been identified since some mechanisms that delineate the dynamics of dispersion of ARGs still remain unclear or not fully understood. This lack is probably due to the complexity and the interconnectedness of the numerous factors involved in this phenomenon concerning host species habits and behavior, spatial and temporal variation in the dissemination of resistant determinants, proximity to humans, livestock and their wastes. The rapid movement of ARGs between taxonomically divergent commensal and pathogenic bacterial strains may pose a threat to food-producing animals sharing similar environments with wildlife and, consequently, to humans. In order to elucidate the missing links for tracking the evolution of AMR, further and in-depth studies are highly needed and, in this regard, we highlight the importance of wildlife rescue and rehabilitation centers as a fundamental component of epidemiological surveillance work.

Moreover, this overview of the literature revealed that non-conventional species that in recent years are becoming popular as pets, although they do not play a major role in the spread of AMR, contribute to delineate the epidemiology and focus on interesting public health topics. The scientific literature on this subject is still scarce and fragmentary and this justifies one of the objectives of this thesis towards the challenge of tackling the AMR through an integrated approach based on the "One Health" concept.

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Chapter 2

Prevalence and antimicrobial resistance of enteropathogenic bacteria in yellow-legged gulls (*Larus michahellis*) in Southern Italy*

*Based on the manuscript: Russo TP, Pace A, **Varriale L**, Borrelli L, Gargiulo A, Pompameo M, Fioretti A, Dipineto L, 2021. Prevalence and Antimicrobial Resistance of Enteropathogenic Bacteria in Yellow-Legged Gulls (*Larus michahellis* in Southern Italy. Animals 11, 275.

2.1 Introduction

The yellow-legged gull (*Larus michahellis*) (Laridae) inhabits different kinds of habitats, from seashores, lakes, and rivers to farmlands and urban surroundings. Indeed, the yellow-legged gull is considered an opportunistic species, given its adaptability to different environments. The Italian yellow-legged gull population, as in most Mediterranean countries, experienced an intense increase (58–125%) during the second half of the 1900s, reaching 45–60 thousand breeding pairs in 2000, from 24–27 thousand pairs estimated in 1983 (Serra et al., 2016). This growth was mainly caused by the mitigation of the negative human impact on gull colonies on one side, and by the increase in trophic sources of human origins (e.g., discarded material or waste) on the other (Serra et al., 2016). As a result, the yellow-legged gull has become a troublesome species all over the Mediterranean area, drawing adverse considerations regarding its interactions with humans and other animals, which can be negatively affected by its aggressive behaviour (Ramos et al., 2009). Gulls are classified as generalist foragers, given their wide range of prey, but they are also considered opportunists, because they can also feed on waste of human origin and carrion (Cockerham et al., 2019). The combination of different habitats and feeding habits makes gulls vulnerable of encountering a wide spectrum of microorganisms (Cockerham et al., 2019). Therefore, gulls have been considered valid sentinel species, especially to explore the influence of urbanization on microbial communities (Fuirst et al., 2018). Indeed, gulls act as potential reservoirs of pathogenic and antibiotic-resistant bacteria and might spread these strains across the different environments they inhabit (Fuirst et al., 2018; Franklin et al., 2020). The dramatic growth of the urban gull populations raises important health concerns, especially considering that the routes of acquisition and dissemination have not been elucidated, thus impeding the development of appropriate control measures (Navarro et al., 2019). Gulls might host several microorganisms, including enteropathogenic bacteria such as *Salmonella*, *Campylobacter* and Shiga toxin-producing *E. coli* (STEC). These bacterial agents have been recognized as mainly responsible for human enteric diseases and have been included by the World Health Organization among those with the evident ability to cause infection in humans following transmission from non-human sources, which should be identified (Makino et al., 2000; EFSA and ECDC, 2018; EFSA, 2020). Indeed, in 2017, *Campylobacter* spp. was reported as the most frequent bacterial cause of human gastroenteritis in Europe since 2005, followed by *Salmonella* spp., *Yersinia* spp., and STEC. Similarly, in 2018, *Salmonella* spp. was reported as the most common food-borne pathogen and the second most frequent zoonotic agent in Europe (EFSA and ECDC, 2018).

Another reason of concern is the overuse of disinfectants and antimicrobials, which have induced a selective pressure on microorganisms during the last decades, acquiring crucial importance when considering bacterial resistance to antibiotics commonly used in human medicine (EFSA, 2020). In this context, antibiotic-resistance might be considered a zoonosis, because resistant strains are transferred among wildlife, domestic animals, and humans, resulting in new reservoirs in the environment and concurring to the amplification and dissemination of antimicrobial resistance (Morè et al., 2017).

Several studies, in different European countries and in the Mediterranean basin, have reported enteropathogenic bacteria in gulls, including resistant strains, and suggested the adoption of these animals as indicators of antibiotic resistance in the environment (Moore et al., 2002; Stedt et al., 2014; Migura-Garcia et al., 2017; Barguigua et al., 2019; Ngaiganam et al., 2019). Given the paucity of information on the subject in southern Italy, the aim of this study was to examine the role of gulls as vectors of zoonotic agents of high importance for human health and as potential reservoirs of antibiotic-resistant strains. Specifically, this study focused on the yellow-legged gull population of the city of Naples, in order to estimate the prevalence of *Salmonella* spp., *Campylobacter* spp., STEC, and *Yersinia* spp., concurrently evaluating the antibiotic resistance of the isolated bacterial strains.

2.2 Matherials and methods

2.2.1 Sampling

From the beginning of April to the end of July, in the four-year period 2016–2019, a total of 225 yellow-legged gulls from the Campania region and recovered at the Wildlife Rescue and Rehabilitation Centre of the University of Naples Federico II were examined. Birds were sampled at their arrival at the centre and a cloacal swab was collected from each animal, using sterile cotton-tipped swabs. Swab samples were placed into 800 µL phosphate-buffered saline (PBS) and transported at 4 °C to the laboratory of the Department of Veterinary Medicine and Animal Productions of the University of Naples Federico II. Sampling procedures are part of the standard clinical evaluation and routine diagnostic testing of recovered wild birds, in accordance with the current legislation (Directive 2010/63/EU).

2.2.2 Bacterial Isolation

Samples were processed in order to isolate *Campylobacter* spp., *Salmonella* spp., STEC, and *Yersinia* spp., following the methods described by the ISO procedures (2017) and Söderlund et al. (2019), with minor modifications (detailed below).
Campylobacter spp.: 100 µL of PBS were transferred into 10 mL of *Campylobacter* selective enrichment broth (Oxoid, UK) and incubated in a microaerophilic atmosphere (8–9% oxygen level and <8% carbon dioxide level, as provided by CampyGen, Oxoid) at 42 °C for 48 h. Subsequently, each sample was plated onto *Campylobacter* blood-free selective agar (CCDA; Oxoid, UK) and incubated in a microaerophilic atmosphere at 42 °C for 48 h. After incubation, the plates were examined for characteristic *Campylobacter* colonies, which were sub-cultured on sheep blood agar (Oxoid, UK) at 42 °C for 24 h. Colonies, after Gram staining, were examined by phase contrast microscopy, and those exhibiting curved or spiral motile rods were submitted to a multiplex polymerase chain reaction (PCR) for species confirmation, as described by Dipineto et al. (2017).
Salmonella spp.: 100 µL of PBS were transferred into 10 mL of Buffered Peptone Water (Oxoid, UK) and incubated at 42 °C for 24 h. Subsequently, an aliquot of each sample was inoculated onto Rappaport-Vassiliadis broth (Oxoid, UK) and incubated at 42 °C for 18 h. After incubation, samples were streaked onto Xylose Lysine Deoxycholate agar (Oxoid, UK) and Brilliant Green Agar (Oxoid, UK), and incubated at 37 °C for 24 h. Suspected colonies were sub-cultured on Rambach agar (Merck) and in Triple Sugar Iron agar (Oxoid, UK) at 37 °C for 24 h and then examined for characteristic *Salmonella* colonies. All *Salmonella* isolated were identified using the miniaturized biochemical system API20E (Biomerieux, Italy).
Yersinia spp.: 100 µL of transport media were pre-enriched for 21 days into 10 mL of PBS at 4 °C. Every seven days, the samples were plated onto *Yersinia* Selective Agar–CIN Medium (Oxoid, UK), incubated at 30 °C for 24–48 h, and examined for characteristic *Yersinia* colonies.
Shiga toxin-producing *E. coli*: 100 µL of PBS were transferred into 10 mL of modified Tryptone Soy Broth (Oxoid, UK), with Novobiocin added (Oxoid, UK). Samples were incubated at 37 °C for 12–18 h and then plated onto Sorbitol MacConkey agar (Oxoid, UK) with added cefixime–tellurite (Oxoid, UK) and onto Sorbitol MacConkey agar with BCIG (Oxoid, UK), both incubated at 37 °C for 18–24 h. Colourless colonies on both media were presumptively identified as *E. coli* O157, whereas coloured colonies on both media were presumptively identified as other *E. coli*. Both colourless and coloured colonies grown on the selective media were subcultured on Nutrient agar at 37 °C for 18–24 h and were sero-grouped on the basis of their O antigen, using anti-coli polyspecific (I, II, III) and monospecific sera (Sifin, Germany), as well as an *E. coli* O157 latex test kit (Oxoid, UK). E. coli results positive to rapid serum agglutination were subcultured on washed sheep blood plates at 37 °C for 18–24 h, and then submitted to multiplex PCR, in order to determine the presence of Shiga toxin (stx1 and stx2) and *E. coli* attaching and effacing (*eae*) genes. DNA extraction and

PCR amplification were performed as previously described (Wang et al., 2002; Dipineto et al., 2010), and PCR products were analysed in a 1.5% agarose gel stained with ethidium bromide (Gibco-BRL, Milan, Italy). A strain of *E. coli* O157 (ATCC 43894) and working solution without DNA were used as positive and negative controls, respectively.

2.2.3 Antimicrobial Susceptibility Testing

Isolated strains were subjected to antimicrobial susceptibility testing, using the disk diffusion technique, in accordance with the criteria established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2018) and the Clinical and Laboratory Standards Institute (CLSI 2012; CLSI, 2015). *Campylobacter* isolates were streaked onto Mueller-Hinton agar with 5% defibrinated sheep blood added (Oxoid, UK), and incubated, with antimicrobial disks, in microaerophilic conditions at 37 °C for 48 h. *Campylobacter* strains were tested with the following antimicrobial agents: azythromicin (AZM, 15 μg), chloramphenicol (CHL, 30 μg), ciprofloxacin (CIP, 5 μg), doxycycline (DO, 30 μg), enrofloxacin (ENR, 5 μg), erythromycin (E, 15 μg), gentamicin (CN, 10 μg), nalidixic acid (NA, 30 μg) and tetracycline (TE, 30 μg). Similarly, *Salmonella* and STEC isolates were streaked onto Mueller-Hinton agar (Oxoid, UK) and then incubated, with antimicrobial disks, at 37 °C for 24 h. The tested antimicrobial agents were: amoxicillin (AMO, 30 μg), amoxicillin–clavulanate (AMC, 20 + 10 μg), ampicillin (AMP, 10 μg), apramycin (APR, 40 μg), ceftazidime (CAZ, 30 μg), chloramphenicol (CHL, 30 μg), ciprofloxacin (CIP, 5 μg), colistin sulphate (CS, 10 μg), doxycycline (DO, 30 μg), enrofloxacin (ENR, 5 μg), gentamicin (CN, 10 μg), nalidixic acid (NA, 30 μg) streptomycin (S, 10 μg), sulphonamides compound (S3, 300 μg), sulphamethoxazole–trimethoprim (SXT, 1.25 + 23.75 μg), and tetracycline (TE, 30 μg). The antibiotics for susceptibility testing were chosen among the most commonly used molecules in human and animal medicine, with available and standardized breakpoints. For all strains the inhibition zones were measured and classified as susceptible, intermediate and resistant, in accordance with the CLSI document (CLSI, 2015). The presence of Extended Spectrum Beta-Lactamase (ESBL)-producing bacteria was evaluated, submitting all strains to the ETEST® ESBL (ESBL CT/CTL 16/1; bioMérieux) and to the combination disk diffusion test, using cefpodoxime (CPD, 10 μg; Oxoid) and cefpodoxime/clavulanic acid (CD, 10/1 μg; Oxoid).

2.3 Results

The bacteriological survey revealed that 93/225 gulls (41.3%; 95% confidence interval (CI) = 34.9–48.1%) were positive for enteropathogenic bacteria, with no co-infection recorded (Table 2.1). Samples processed for *Yersinia* spp. were consistently negative.

Table 2.1. Prevalence of enteropathogenic bacteria isolated from 225 yellow-legged gulls.

Bacterial species	Positive Animals (n.)	Prevalence (95% CI*)	Identification	Strains (n)
Campylobacter spp.	60	26.7% (21.4%-32.8%)	*C. coli* *C. jejuni*	36/60 24/60
Salmonella spp.	3	1.3% (0.5%-3.8%)	*S. arizonae*	3/3
STEC	30	13.3% (9.5%-18.3)	*E. coli* O128 *E. coli* O26 *E. coli* O157 *E. coli* O11	12/30 9/30 6/30 9/30

*CI, Confidence interval.

Campylobacter spp. were isolated from 60/225 (26.7%; 95% CI = 21.4–32.8%) samples. Among these, as confirmed by multiplex PCR, 36/60 (60%) were identified as *C. coli* and 24/60 (40.0%) were identified as *C. jejuni*. All *Campylobacter* tested were susceptible to chloramphenicol and gentamicin (Table 2.2). The main antimicrobial resistances detected for *C. jejuni* and *C. coli*, were to tetracycline (62.5% and 52.8%, respectively), ciprofloxacin (37.5% and 33.3%, respectively) and nalidixic acid (37.5% and 27.7%, respectively).

Table 2.2. Antibiotic resistance of 60 strains of Campylobacter spp., isolated from 225 yellow-legged gulls.

Strain	No. of Resistant Strains to Tested Antibiotics (%)								
	AZM	CHL	CIP	CN	DO	E	ENR	NA	TE
C. coli (n. 36)	8 (22.2)	0	12 (33.3)	0	6 (16.6)	4 (11.1)	11 (30.5)	10 (27.7)	19 (52.8)
C. jejuni (n. 24)	6 (25.0)	0	9 (37.5)	0	5 (20.8)	4 (16.0)	7 (29.1)	9 (37.5)	15 (62.5)

AZM = azythromicin, 15 µg; CHL = chloramphenicol, 30 µg; CIP = ciprofloxacin, 5 µg; CN = gentamicin, 10 µg; DO = doxycycline, 30 µg; E = erythromycin, 15 µg; ENR = enrofloxacin, 5 µg; NA = nalidixic acid, 30 µg; TE = tetracycline, 30 µg.

When considering resistance to multiple antibiotics, among the 36 *C. coli* strains, nine (25.0%) were simultaneously resistant to tetracycline and ciprofloxacin; three (8.3%) were simultaneously resistant to tetracycline, ciprofloxacin, and erythromycin; and three (8.3%) were also resistant to azythromicin and nalidixic acid, in addition to the previous antibiotics. Among the 24 *C. jejuni* strains, six (25.0%) were simultaneously resistant to azythromicin, ciprofloxacin and tetracycline; four (33.3%) were simultaneously resistant to azythromicin, ciprofloxacin, tetracycline, and erythromycin; and two (8.3%) were also resistant to nalidixic acid, in addition to the previous antibiotics.

Salmonella spp. were isolated from 3/225 samples (1.3%; 95% CI 0.5–3.8%), and all strains were identified as *Salmonella arizonae*. One strain was susceptible to all tested antibiotics, whereas the other two strains (66.6%) were resistant only to sulphonamides compound.
E. coli were isolated from 189/225 samples (84.0%; 95% CI 78.7–88.2%), but Shiga toxin-producing *E. coli* were recovered only in 30/225 gulls (13.3%; 95% CI 9.5–18.3%) and classified according to their O antigen as follows: O128 (n = 12, 40.0%) O26 (n = 9, 30.0%), O157 (n = 6, 20%) and O11 (n = 3, 10.0%). The other strains that presented a non-typable O antigen were considered generic *E. coli* and were not further analysed. Multiplex PCR showed that all 30 strains carried one or more virulence genes (*stx*1 = 17; *stx*2 = 16; *eae* = 21). As detailed in Table 2.3, the most frequently detected resistances were towards tetracycline (56.6%), followed by ampicillin (50.0%) and ciprofloxacin (33.3%), whereas all strains were susceptible to chloramphenicol (Table 2.3). The majority of STEC isolates (76.6%) were simultaneously resistant to at least two antibiotics, and nine isolates (30.0%) displayed simultaneous resistance to at least three antibiotics. Specifically, eleven (36.6%) STEC isolates were resistant to ampicillin and tetracycline; four (13.3%) were resistant to ampicillin, tetracycline and enrofloxacin; and two (6.67%) were resistant to ampicillin, tetracycline, and sulphamethoxazole–trimethoprim. Only two (6.67%) *E. coli* O26 strains were positive to the ESBL test.

Table 2.3. Antibiotic resistance of 30 strains of Shiga toxin-producing E. coli, isolated from 225 yellow-legged gulls.

No. of Resistant Strains to Tested Antibiotics (%)															
AMP	AMO	AMC	APR	CAZ	CIP	CHL	CS	DO	ENR	CN	NA	S	TE	SXT	ESBL+
15	5	4	4	1	10	0	3	4	5	4	3	1	17	8	2
(50.0)	(16.6)	(13.3)	(13.3)	(3.3)	(33.3)		(10.0)	(13.3)	(16.6)	(13.3)	(10.0)	(3.3)	(56.6)	(26.6)	(6.6)

AMP = ampicillin, 10 µg; AMO = amoxicillin, 30 µg; AMC = amoxicillin–clavulanate, 20 + 10 µg; APR = apramycin, 40 µg; CAZ = ceftazidime, 30 µg; CIP = ciprofloxacin, 5 µg; CHL = chloramphenicol, 30 µg; CS = colistin sulphate, 10 µg; DO = doxycycline, 30 µg; ENR = enrofloxacin, 5 µg; CN = gentamicin, 10 µg; NA = nalidixic acid, 30 µg; S = streptomycin, 10 µg; TE = tetracycline, 30 µg; SXT = sulphamethoxazole–trimethoprim, 1.25 + 23.75 µg; ESBL+ = Extended Spectrum Beta-Lactamase production.

2.4 Discussion

Enteropathogenic bacteria were detected in 41.3% of the yellow-legged gulls examined in the present survey, including *Salmonella* spp., *Campylobacter* spp., and Shiga toxin producing *E. coli*. No co-infections were recorded, similarly to a previous survey, focused on two of these enteropathogenic bacteria (Morè et al., 2017).

Various studies have previously investigated the prevalence of *Campylobacter* spp. in gulls worldwide, but the results are many and heterogeneous. The prevalence of *Campylobacter* spp. reported here (26.7%; 95% CI 21.4–32.8%), as well as the most frequently identified species, differ from other studies (Morè et al., 2017; Migura-Garcia et al., 2017; Broman et al., 2002). Migura-Garcia et al. detected 19 *Campylobacter* isolates from 9.3% chicks of yellow-legged gull along the north-eastern Iberian coast and identified them as *C. jejuni* (65.0%) and *C. lari* (35.0%). Similarly, Broman et al. isolated 250 *Campylobacter* species from 31.8% black-headed gulls (*Chroicocephalus ridibundus*) in southern Sweden, with *C. jejuni* as the most prevalent species (94.0%), followed by *C. lari* (3.2%) and *C. coli* (2.8%). In another survey, Moré et al. isolated thermophilic *Campylobacter* from 12.4% kelp gull chicks (*Larus dominicanus*) in South Africa, with *C. jejuni* as the most frequently identified species, followed by *C. lari*. In the aforementioned studies, *C. jejuni* was predominant, whereas *C. lari* and *C. coli* were less frequently detected. Contrarily, Campylobacter strains isolated in our study were identified mainly as *C. coli* (60.0%) and *C. jejuni* (40.0%), whereas *C. lari* was never identified. This contrast might be explained by different gull species and age classes, or by the influence of geographical circumstances, living conditions, feeding habits, and the use of refuse dumps (Ramos et al., 2010).

Concerning antimicrobial resistance, all tested *Campylobacter* strains exhibited susceptibility to chloramphenicol and gentamicin, whereas different rates of resistance were detected towards tetracyclines (16.6–62.5%), fluoroquinolones (29.1–37.5%) and macrolides (11.1–25%). Additionally, 22 strains showed multidrug resistance, defined as resistance to at least three classes of antimicrobial agents (Magiorakos et al., 2012). These data are in line with previous surveys that reported resistances towards tetracyclines and fluoroquinolones, although *Campylobacter* resistance to erythromycin, and multidrug resistance, were not reported (Morè et al., 2017; Migura-Garcia et al., 2017). Indeed, the pattern of resistances as detected in our study is particularly relevant, because macrolides represent the first-line therapy for human *Campylobacter* infections, and tetracycline and fluoroquinolones are considered valid alternatives (García-Fernández et al., 2018).

Salmonella spp. was isolated from only three birds, resulting in a lower prevalence (1.3%) as compared to previous studies (Migura-Garcia et al., 2017; Ramos et al., 2010), and identified as *Salmonella enterica arizonae*, although the subspecies *S. enterica enterica* has been more commonly described (Morè et al., 2017; Ramos et al., 2010). Curiously, we found a higher occurrence of *Campylobacter* than *Salmonella*, in contrast with the pattern observed in kelp gulls from South Africa, but similar to the greater crested terns examined in the same study (Morè et al., 2017). Actually, the prevalence of *Salmonella* in gulls appears variable, and our results are analogous to those reported by Palmgren et al. (2006) in black-headed gulls from southern Sweden. As suggested for *Campylobacter*, the differences might be related to distinct locations of colonies and especially to different feeding habits (Ramos et al., 2010). Gulls are challenged by conditions raised by humans; therefore, these birds might come into contact with contaminated environments, such as surface water polluted by farm effluents, or sewage (Manfreda et al., 2006; Dumontet et al., 2001). Another explanation which could be explored is the presence of other potential reservoirs, raised by humans or wild (e.g., chickens, pigeons, corvids, etc.), sharing the same environment inhabited by the gulls (Söderlund et al., 2019¸ Manfreda et al., 2006; Dumontet et al., 2001; Dudzic et al., 2016).

Antimicrobial resistance of *Salmonella* has been previously reported, mainly towards tetracyclines and streptomycin (Morè et al., 2017; Migura-Garcia et al., 2017). However, all three *Salmonella* strains isolated here exhibited susceptibility to all the tested antimicrobials, excluding sulphonamides, similarly to other studies (Morè et al., 2017; Palmgren et al., 2006). This is surprising for an urban

area such as the city of Naples, because a link has been suggested between the use of urban refuse as a food source and the occurrence of enteric antibiotic-resistant bacteria in gulls (Migura-Garcia et al., 2017; Ramos et al., 2010).

Shiga toxin-producing *E. coli* have been largely detected in domestic and wild animals, including gulls (Makino et al., 2000; Barguigua et al., 2019). Although the prevalence was lower, as compared to other surveys conducted on gulls across Europe (Stedt et al., 2014), the STEC percentage is in line with other studies conducted on wild birds recovered in urban surroundings (Fadel et al., 2017; Morabito et al., 2001). Unfortunately, it was not possible to establish the O:H serotypes, in order to determine the seropathotypes according to Karmali et al. (2003). However, all of our strains carried one or more STEC-associated genes, highlighting the potential role of gulls as a source of STEC to other animals and humans (Makino et al., 2000; Barguigua et al., 2019).

The vast majority (76.6%) of STEC isolated here were simultaneously resistant to at least two antibiotics, and 30.0% of the strains exhibited simultaneous resistance to at least three antibiotics, raising important public health concerns (Stedt et al., 2014; Barguigua et al., 2019). The most commonly detected STEC resistances were towards tetracycline (56.6%) ampicillin (50.0%) and ciprofloxacin (33.3%), data that are in accordance with other studies on gulls, such as one described in Spain, where more than half of the strains exhibited antibiotic resistance (Stedt et al., 2014). Actually, Stedt et al. (2014) highlighted a south-to-north gradient in Europe (valid for *E. coli* from humans, food production animals and gulls), characterized by higher levels of resistance in Mediterranean countries and lower levels of resistance in northern Europe, with few local variations. These results, including ours, reflect the overuse of antibiotics in veterinary and human medicine over the years (Stedt et al., 2014; Barguigua et al., 2019), and suggest that gulls might act as vectors but also as victims, acquiring resistant strains that potentially originate from humans or animals, which are often subjected to antibiotic administration (Miles et al., 2006; Musa et al., 2020).

Yersinia spp. has never been isolated, although this microorganism has been previously isolated from gulls (Niskanen et al., 2020). However, the strains identified in that study were characterized by low virulence and pathogenicity, posing little or no risk to animal and human health.

Anthropic activities and gull habits seem to be the main factors involved in the dissemination of resistant bacteria among gulls and other animals, humans, and the environment (Stedt et al., 2014; Barguigua et al., 2019). In the present research, an important role may have been played by the presence of open landfill sites, which are widespread in the study region. Therefore, similarly to other animals inhabiting the marine environment, the occurrence of resistant bacteria in gulls represents an indication of anthropic pressure on the environment and the antibiotic resistome (Blasi et al., 2020; Alduina et al., 2020).

The present study indicates that yellow-legged gulls might act as reservoirs or carriers of enteropathogenic bacteria, contributing to the epidemiological distribution of such pathogens as well as the potential maintenance and spread of antimicrobial resistance, with potential risks of antibacterial efficacy in human and animal medicine. The reason might be associated to the close contact between gulls and human activities, in particular through parks and shores, which serve as meeting spots for humans, domestic animals, synanthropic birds, and environmental waters. Our findings might be particularly important for other coastal urbanized areas, where gull populations have experienced a significant increase, and where these birds, or their droppings, might come into contact with humans, especially those belonging to the most susceptible age groups (e.g., infants, the elderly, people with immunodeficiencies, etc.), thereby increasing the risk of infection. Further research should be conducted in order to investigate and elucidate the routes through which these bacteria are circulating.

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Chapter 3

Antimicrobial resistance of *Escherichia coli* and *Pseudomonas aeruginosa* from companion birds*

*Based on the manuscript: **Varriale L**, Dipineto L, Russo TP, Borrelli L, Romano V, D'Orazio S, Pace A, Menna LF, Fioretti A, Santaniello A, 2020. Antimicrobial Resistance of *Escherichia coli* and *Pseudomonas aeruginosa* from Companion Birds. Antibiotics 9, 780.

3.1 Introduction

Antimicrobial resistance (AMR) is a serious concern compromising the empirical treatment of infections and resulting in a lack of effective antibiotics and high medical expenses (Founou et al., 2017).
The rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives. Many decades after the first patients were treated with antibiotics, bacterial infections have again become a threat. The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry due to reduced economic incentives and challenging regulatory requirements (Ventola, 2015; de Kraker et al., 2016). The US Center for Disease Control and Prevention (CDC), the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO) are classifying infections caused by multidrug-resistant (MDR) bacteria as an alarming global disease and a worldwide public health problem (CDC, 2020; ECDC, 2019; WHO, 2017; Tacconelli et al., 2018). Because of the abuse and misuse of antibiotics both in humans and livestock, and the consequent release in the environment, selected microorganisms have acquired resistance over time by mutation or horizontal transfer of mobile genetic elements carrying resistance genes. The detection of specific mechanisms of resistance by molecular methods has become crucial, both from an epidemiological and a therapeutical point of view. The discovery of novel molecules as an alternative strategy to antibiotics represents a high priority, as well as other important measures of surveillance and control (Roca et al., 2015; Garcia-Migura et al., 2014). Coordinated efforts to implement new policies, renew research efforts, and pursue steps to manage the crisis are urgently needed (Dipineto et al., 2017).
Several studies have been conducted on livestock showing the correlation between the systemic use of antibiotics and the onset of resistant bacterial strains (WHO, 2017). In contrast, although companion birds are historically considered an excellent animal reservoir, poor information on the antimicrobial resistance in these species is available in the literature (Dipineto et al., 2017). This topic has been largely investigated in wild birds, specifically in birds of prey, waterfowl and passerines, which seem to be a remarkable reservoir of multi-resistant *E. coli* strains, representing a notable risk for human and animal health by the spread of these bacteria to waterways and other environmental sources via their fecal deposits (Benskin et al., 2009; Guenther et al., 2010).
In Italy, according to data from the Assalco-Zoomark report (2017), pet birds are about thirteen million, almost twice the number of dogs and cats. In pet bird farms, the administration of antibiotics without medical control is a very common practice, contributing to the increasing resistance rates.
In the light of the above considerations, this study was aimed at evaluating antimicrobial resistance of *Escherichia coli* and *Pseudomonas aeruginosa* isolated from companion birds to better understand the epidemiological role of these species in the spread of multidrug resistant bacteria between animals, humans and the environment.

3.2 Materials and Methods

3.2.1 Sampling

During the period January 2016–December 2018, cloacal swabs were collected from a total of 735 clinically healthy birds. Sampled animals belonged to the following families and species: Fringillidae (*Carduelis carduelis*, *Serinus canaria*), Estrildidae (*Erythrura gouldiae*, *Lonchura striata domestica*, *Taeniopygia guttata*), Psittacidae (*Melopsittacus undulatus*, *Agapornis roseicollis*) and Columbidae (*Columba livia domestica*). All the birds analyzed were selected from different farms located in Campania region (southern Italy), separated by species and kept in cages. As stated by their respective owners, no birds were on antibiotic treatment at the time of sampling and had not received antibiotic treatments in the previous three months. Samples were collected by sterile cotton-tipped swabs. Each sample was stored in Amies Charcoal Transport Medium (Oxoid, Basingstoke, United Kingdom) at 4 °C, transported to the laboratory, and analyzed within 2 h of collection. Furthermore, twenty birds of prey (*Buteo buteo*, *Accipiter gentilis*, *Falco peregrinus*) housed for falconry were also sampled as described previously.

3.2.2 Bacterial isolation

Cloacal samples were inoculated into Buffered-Pepton Water (BPW, Oxoid, Milan) and incubated at 37 °C for 24 h. Cultures obtained were plated onto MacConkey agar and Cetrimide agar (Oxoid, Milan), and incubated at 37 °C for 24 h. Suspected *E. coli* colonies were streaked onto Tryptone Bile X-Glucuronide and incubated at 42 °C for 24 h. Finally, all isolates were biochemically identified by using API 20E system (BioMèrieux, Marcy l'Etoile, France), whereas potential *Pseudomonas* spp. colonies were submitted to oxidase test and processed by biochemical identification by API 20 NE system (BioMèrieux, Marcy l'Etoile, France). In addition, all laboratory procedures were carried out according to UNI EN ISO 9001:2015 (Cert. N. 317jSGQ10).

3.2.3 Antimicrobial Susceptibility Testing

All isolates were submitted to antimicrobial susceptibility testing using the disc diffusion method according to Clinical Laboratory Standards Institute (CLSI, 2012). The antimicrobials tested were amoxycillin/clavulanic acid (AMC; 30 μg), sulfamethoxazole-trimethoprim (SXT; 25 μg), doxycycline (DO; 30 μg), enrofloxacin (ENR; 5 μg), gentamicin (CN; 10 μg), and oxytetracycline (OT; 30 μg). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC15442 were used as control strains in each experiment. The inhibition zones were measured and scored as sensitive, intermediate susceptibility and resistant according to the CLSI documents (CLSI, 2012; CLSI, 2014).

3.3 Results

A total of 59/755 (7.8%, 95% confidence interval = 6.05–10.02%) samples were positive for *P. aeruginosa*, whereas a total of 231/755 (30.6%, 95% CI = 27.35–34.04%) samples were positive for *E. coli*. Additionally, a few strains of Gram-negative bacteria such as *Pantoea* spp., *Serratia* spp, *Morganella* spp., and *Citrobacter* spp. were occasionally isolated.

With respect to *P. aeruginosa*, 45/59 (76.3%) strains were resistant to amoxycicillin/clavulanic acid (AMC; 30 µg) and to sulfamethoxazole-trimethoprim (SXT; 25 µg), 42/59 (71.2%) were resistant to doxycycline (DO; 30 µg), 46/59 (78%) were resistant to enrofloxacin (ENR; 5 µg), 17/59 (28.9%) were resistant to gentamicin (CN; 10 µg) and 48/59 (81.3%) were resistant to oxytetracicline (OT; 30 µg). The majority of strains showed multidrug resistance.

Among the *E. coli* isolates, 118/231 (51.1%) were resistant to amoxycicillin/clavulanic acid (AMC; 30 µg), 127/231 (55%) were resistant to sulfamethoxazole-trimethoprim (SXT; 25 µg), 132/231 (57.1%) were resistant to doxycycline (DO; 30 µg), 92/231 (40%) were resistant to enrofloxacin (ENR; 5 µg), 61/231 (26.4%) were resistant to gentamicin (CN; 10 µg), 147/231 (63.6%) were resistant to oxytetracicline (OT; 30 µg). For *E. coli*, most strains were also multidrug resistant. Results are summarized in Tables 3.1 and 3.2.

Table 3.1. Prevalence of P. aeruginosa strains and percentage of AMR phenotypes in the examined animals.

Examined Animals (Number)	Acronym for Antibiotics Number of Positive Samples (%)					
	AMC	SXT	DO	ENR	CN	OT
Fringillidae (388)	38/45 (84.4)	37/45 (82.2)	37/45 (82.2)	40/45 (88.8)	14/45 (31.1)	39/45 (86.6)
Estrildidae (52)	4/5 (80.0)	4/5 (80.3)	1/5 (20.0)	1/5 (20.0)	0/5 (0.0)	4/5 (80.0)
Psittacidae (77)	0/3 (0.0)	1/3 (33.3)	1/3 (33.3)	2/3 (66.7)	2/3 (66.7)	2/3 (66.7)
Columbidae (218)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)
Birds of prey (20)	2/5 (40.0)	2/5 (40.0)	2/5 (40.0)	2/5 (40.0)	0/5 (0.0)	2/5 (40.0)

AMC: Amoxycillin/Clavulanic Acid 30µg; SXT: Trimethoprim/Sulfamethoxazole 25 µg; DO: Doxycycline 30 µg; ENR: Enrofloxacin 5 µg; CN: Gentamicin 10 µg; OT: Oxytetracycline 30µg.

Table 3.2. Prevalence of E. coli strains and percentage of AMR phenotypes in the examined animals.

Examined Animals (Number)	Acronym for Antibiotics Number of Positive Samples (%)					
	AMC	SXT	DO	ENR	CN	OT
Fringillidae (388)	24/33 (72.7)	13/33 (39.4)	15/33 (45.4)	18/33 (54.5)	12/33 (36.4)	18/33 (54.5)
Estrildidae (52)	5/13 (38.5)	4/13 (30.8)	4/13 (30.8)	4/13 (30.8)	2/13 (15.4)	6/13 (46,1)
Psittacidae (77)	7/8 (87.5)	8/8 (100)	7/8 (87.5)	8/8 (100)	3/8 (37.5)	7/8 (87.5)
Columbidae (218)	77/162 (47.5)	100/162 (61.7)	106/162 (65.4)	62/162 (38.3)	42/162 (25.9)	114/162 (70.4)
Birds of prey (20)	5/20 (33.3)	2/20 (13.3)	0/20 (0.0)	0/20 (0.0)	2/20 (13.3)	2/20 (13.3)

AMC: Amoxycillin/Clavulanic Acid 30µg; SXT: Trimethoprim/Sulfamethoxazole 25 µg; DO: Doxycycline 30 µg; ENR: Enrofloxacin 5 µg; CN: Gentamicin 10 µg; OT: Oxytetracycline 30µg.

3.4 Discussion

In this study *E. coli* and *P. aeruginosa* were the most frequently isolated bacteria from companion birds with a prevalence of 30.7% and 7.8%, respectively. These microorganisms are the most abundant facultative bacterial species in the normal microbiota of the large intestine of animals and humans (Lister et al., 2009; Tenaillon et al., 2010; Singleton, 1999). Particularly, *E. coli* is one of the most pathogenic bacterial species in cage birds causing aerosacculitis, polyserositis, septicemia and other mainly extraintestinal diseases (Dho-Moulin and Fairbrother, 1999), whereas *P. aeruginosa* is ubiquitous in aviaries and, under favorable conditions, acts as an opportunistic pathogen. It may occur with localised infections such as rhinitis, sinusitis and laryngitis or can be associated with septicemia and hemorrhagic enteritis. Enrofloxacin, gentamicin, ceftazidime, amikacin and piperacillin are the most common antimicrobials used as treatment (Bailey et al., 2000). Antibiotic resistance of Gram-negative bacteria has been largely reported in avian medicine, especially in poultry, but data available in companion birds are very scant. Our findings are consistent with those reported by Sigirci et al. (2020), who isolated *E. coli* from 37.7% of the examined companion birds, with the majority of the isolates resistant to tetracycline (84%) followed
by sulfamethoxazole/trimethoprim (46%), streptomycin (34%), and kanamycin (25%). Furthermore, Di Francesco et al. (2020), evaluated the AMR of Gram-negative species isolated from 456 domestic canaries, showing multiple resistance, especially against amoxycillin, erythromycin, spiramycin, tiamulin, and tylosin. Our results are in line with this study regarding the multidrug resistance exhibited by *E. coli* strains isolated from canaries, but not for the antimicrobials, which were amoxycillin/clavulanic, sulfamethoxazole-trimethoprim, doxycycline, enrofloxacin, gentamicin and oxytetracycline.

E. coli is usually isolated in the gut of healthy pigeons and in many studies is considered as an antibacterial resistance indicator. Ghanbarpour et al. (2020), characterized the AMR genotypes in relation with phenotypic traits of *E. coli* strains isolated from household pigeons. Approximately half of the isolates were resistant to three or more antibiotics (40.1%), in particular to tetracycline (98%), cefotaxime (49.3%), kanamycin (34.2%), trimethoprim-sulphamethoxazole (28.2%), enrofloxacin (17.1%), gentamicin (11.1%) and florfenicol (7.8%). According to these findings, our study showed a high prevalence of MDR *E. coli* strains, with the highest rates for oxytetracycline (70.4%) and trimethoprim-sulfamethoxazole (61.7%). Oxytetracycline, florfenicol and trimethoprim-sulfamethoxazole are the most common molecules administered to pigeons in the treatment of respiratory, digestive and pyogenic infections, contributing to the occurrence of MDR strains, in addition to other environmental sources (Dolka et al., 2020). Domestic pigeons are likely to harbour resistance genes which could transfer directly or indirectly to humans and animals, and from a One Health perspective this risk should not be underestimated.

A study conducted by Carroll et al. (2015), assessed the antimicrobial resistance of *Escherichia coli* in wild birds. Among the examined species, starlings showed the highest prevalence of AMR (5.4%), with tetracycline and streptomycin as predominant resistant phenotypes. Even if molecular processing highlighted that all isolates belonged to phylogenetic group B1, this commensal *E. coli* group is often responsible for both intestinal and extraintestinal infections in different animal species. Commensal *E. coli* strains may harbour antimicrobial resistance determinants, act as reservoirs of resistance, and at a later stage transfer these resistance features to pathogenic bacteria. Even if wild birds are not exposed to antibiotics directly, contact with sewage or animal manure could explain the acquisition of resistance genes and especially migratory birds can contribute to their pandemic spread. Pandemic ESBL-producing *E. coli* clones or plasmids shared by humans, domestic animals, and wildlife further strengthen this hypothesis (Wang et al., 2017). With respect to *E. coli* strains isolated in our study from birds of prey (75%), our results are not completely in accordance with those of Giacopello et al. (2016), who isolated *E. coli* (53.1%) in raptors admitted to a wildlife rescue center and reported trimethoprim/sulfamethoxazole as the most frequent resistance displayed by Enterobacteriaceae

(83.7%), whereas we mentioned amoxycillin/clavulanic acid (33%) as the highest percentage of AMR.

MDR phenotypes are frequently encountered in *P. aeruginosa* causing nosocomial infections. This opportunistic pathogen is considered as "critical" and the identification of new antibiotics is essential to overcome its MDR properties (Pragasam et al., 2018). To our knowledge, data on the AMR profiles of this microorganism in companion birds are not available, even if the possibility to transmit infections and resistant traits to other species, humans included, is a public health concern that should require more attention. Our results show that 7.8% of the examined birds were positive for *P. aeruginosa*, with all the strains resistant to at least one antibiotic and the majority showing multidrug resistance with rates up to 100%. In a study conducted by Vidal et al. (2017), *P. aeruginosa* (7%) was detected in birds of prey with systemic infection and oral lesions, whereas in our study the prevalence was higher (25%), even if the raptors appeared clinically healthy. Most strains displayed resistance to all the antimicrobials tested, except one to gentamicin. On the contrary, the above-mentioned study reported 100% resistance to clindamycin and 21% to gentamicin.

Based on our findings, we highlight the role of companion birds as potential vectors and reservoirs in the spread and transmission of AMR and emphasize the importance of surveillance programs to prevent the impact of this threat on public health (Berendonk et al., 2015). We would also encourage further, deeper studies in this field to be conducted in order to characterize the plasmid profiles eventually associated with the multidrug resistant phenotypes. In fact, identifying the specific mechanisms that underlie antimicrobial resistance in these species would provide additional information about their role in the animal-human-ecosystem interface.

In our opinion, a One Health approach is increasingly needed, which involves collaboration between veterinarians, doctors, public health professionals and epidemiologists, and may represent a more effective and shared opportunity for the global challenge to AMR that currently affects humans, animals and the ecosystem in a transversal way.

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Chapter 4

Bacteria and parasites in *Podarcis sicula* and *P. sicula klemmeri**

*Based on the manuscript: Dipineto L, Raia P, **Varriale L**, Borrelli L, Botta V, Serio C, Capasso M, Rinaldi L, 2018. Bacteria and parasites in *Podarcis sicula* and *P. sicula klemmeri.* BMC Vet Res 14, 392

4.1 Introduction

The Italian wall lizard, *Podarcis sicula*, is one of the most common lizards in Italy (Corti et al., 2006). The snout-vent length (SVL) is, on average, 15 to 25 cm long. The colour pattern is characterized by a green or brown back and whitish belly, although melanic variants, with either shorter, or longer SVL, are known to occur in several islands inthe Mediterranrean sea. As a common, and easily managed study model, the Italian wall lizard was subjected to several studies, regarding aspects as disparate as phenotypic response to predation (Vervust et al., 2007), feeding behaviour (Capula et al., 2011), ontogeny (Piras et al., 2011), adaptation to novel environments (Kapsalas et al., 2016) biogeography (Podnar et al., 2005; Senczuk et al., 2017), and its role as a biological indicator (De Falco et al., 2014).
Podarcis sicula klemmeri (Lanza and Capolongo, 1972) is one of the subspecies belonging to *P. sicula*. It is confined to a small, 1 km2 large islet, Licosa, off the western coast of Italy. As with many other insular populations of the Italian wall lizard, *P. s. klemmeri* is melanic, meaning the back appears tinged with blue, and the pale underside is bluish as well, rather than the usual white of continental populations.
Melanic variants have been investigated for the so-called Island syndrome (Adler and Levins, 1994), which is a suite of phenotypic character shifts in insular populations including, besides melanism, changed body size, feeding behaviour and ecology, patterns of aggressiveness, and life history traits (Meiri, 2007; Raia et al., 2010; Raia et al., 2010a; Novosolov et al., 2013; Cooper et al., 2015). The link between melanism and characters shifts on islands was found to be in the activity levels of melano-cortin receptors, MCRs (Raia et al., 2010a). Melanocortins form a suite of five receptors activated pleyotropically by a single DNA locus, the proopiomelanocortin POMC gene, that happen to regulate feeding and sexual activity, immunocompetence and body colour (Ducrest et al., 2008; Emaresi et al., 2013; Kim et al., 2013). Interestingly, Monti et al. (2013) tested whether *P. s. klemmeri* individuals have different ectoparasite loads (i.e., the density of ticks and mites on the skin) as compared to the continental individuals. They found reduced load in insular individuals, consistently with their comparatively higher α-melanocyte-stimulating hormone (MSH) levels.
Herein, we deepen microbiological and parasitological investigations on *P. s. klemmeri*. Besides a few studies (Casanova et al., 2003; Lhermitte et al., 2008), these aspects have not been scrutinized so far yet remain very important in the case of melanic insular lizards, whose immune system is expected to be depressed by great investment in reproduction, and life at high density (Raia et al., 2010a; Monti et al., 2013; Novosolov et al., 2016). This study was undertaken with the aim to evaluate the presence of potentially zoonotic bacteria and parasites in wild-caught insular individuals of *P. sicula klemmeri*, along with mainland individuals of *P. sicula*.

4.2 Materials and Methods

4.2.1 Study animals

From November 2015 to May 2016, we collected and examined, within 24 h from capture, a total of 24 individuals, 20 (14 males and 10 females) belonging to the subspecies *P. sicula klemmeri*, and 4 individuals belonging to the species *P. sicula*. The *P. sicula klemmeri* specimens were from the small islet of Licosa, whereas the *P. sicula* specimens from Punta Licosa mainland (Campania region of southern Italy). The study site, collection and housing methods were described in Raia et al. (2010, 2010a). Lizards captured on Licosa islet were identified with the code "LIC" and a progressive number (from LIC 1001 to 1020); the lizards from Licosa mainland were identified with the code "PLIC" and a progressive number (from PLIC 1001 to 1004).
In order to perform the microbiological analysis, oral and cloacal samples were individually collected by sterile cotton-tipped swabs and then inoculated in Phosphate Buffered Saline (PBS). For the parasitological analyses, different methods were used: faecal samples were collected from each lizard, scotch tape test was performed on the skin of each animal and, eventually, a necropsy with stereo microscope was carried out on two lizards found dead on the insular study site. After sampling, animals were released in the same area where they were captured. All experiments described were performed in accordance with the local and national guidelines governing animal experiments (86/ 609/CEE and its modifications).

4.2.2 Microbiological and parasitological analyses

Oral and cloacal swabs stored in PBS were inoculated in buffered peptone water (BPW), *Campylobacter*-selective enrichment broth (CSEB), cooked meat medium (CMM), modified tryptone soy broth (MTSB). The samples inoculated in BPW were incubated at both 25 °C and 37 °C for 24 h and then inoculated/streaked into Rappaport-Vassiliadis broth (RV), Columbia blood agar base (CBA; Oxoid, Milan, Italy), *Pseudomonas* cetrimide agar (PCA; Oxoid), MacConkey agar (MCA; Oxoid) and Baird-Parker agar (BPA; Oxoid). The samples inoculated in MTSB were incubated at both 25 °C and 37 °C for 24 h and then spread on sorbitol MacConkey agar (SMCA; Oxoid). The samples inoculated in CSEB were incubated in microaerophilic atmosphere at both 25 °C and 42 °C for 48 h and then streaked on *Campylobacter* blood-free selective agar (CBFA; Oxoid). Instead, the samples inoculated in CMM were incubated in anaerobic atmosphere at both 25 °C and 37 °C for 24 h, and then streaked on anaerobe basal agar (ABA; Oxoid). The remaining PBS were incubated at 4 °C for 14 days, spread on *Yersinia* selective agar base (cefsulodin-irgasan-novobiocin, CIN Agar; Oxoid) and incubated at 30 °C for additional 24–48 h. CBA, PCA, MCA, SMCA, CEOA and BPA were incubated at both 25 °C and 37 °C for 24–48 h, whereas RV was incubated at both 25 °C and 42 °C for 24–48 h and then streaked on xylose lysine desoxycholate agar (XLD) and brilliant green agar (BGA); CBFA were incubated in microaerophilic atmosphere at both 25 °C and 42 °C for 24–48 h, ABA plates were anaerobically incubated both at 25 °C and 37 °C for 48 h and checked daily for a further week before discarding. All the isolates were previously identified according to their morphological features, their growing need, Gram colouring, motility and pigments production test and through conventional biochemical and phenotypic test. Progressively, the isolates were identified through the biochemical systems API 20 E, API 20 NE (bioMerieux, Mercy-l'Etoile, France) and RapID ANA II, RapID NF PLUS, RapID STAPH PLUS (Oxoid). *Escherichia coli* strains were serogrouped with antisera poly- and monospecific (Sifin) whereas, suspected *Campylobacter* strains were identified by PCR as reported by Gargiulo et al. (2008).
In order to detect and count parasitic elements (eggs, larvae, cysts, oocysts), each faecal sample collected from the 24 lizards was analysed with the FLOTAC pellet technique (Rinaldi et al., 2012; Cringoli et al., 2010). The analytic sensitivity of the FLOTAC pellet technique varied on the basis of the weight of each pellet μμ (Rinaldi et al., 2012) and expressed as eggs/oocysts per gram (EPG/OPG)

of faeces. Two flotation solutions were used: sodium chloride (NaCl, specific gravity = 1200) and zinc sulfate (ZnSO4, specific gravity = 1350) to detect protozoa/nematoda and trematoda, respectively. The scotch tape test was performed on the skin of the lizards, specifically, at the level of skin folds, around the eye, the cloaca and the eardrum recesses. The necropsy of the two lizard carcasses was performed under a stereomicroscope using the reptile necropsy protocol reported in Jacobson (1978).

4.2.3 Antimicrobial Susceptibility testing

Pseudomonas aeruginosa, *Escherichia coli* O145, *Citrobacter* spp., *Enterobacter* spp. isolates were submitted to antimicrobial susceptibility testing using the disc diffusion method according to Clinical Laboratory Standard Institute (CLSI, 2012). The antimicrobials tested were ampicillin (10 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), sulphamethoxazole-trimethoprim (25 µg), nalidixic acid (30 µg), amoxicillin-clavulanic acid (30 µg), doxycycline (30 µg), gentamicin (10 µg), streptomycin (10 µg), amikacin (30 µg), nitrofurantoin (30 µg), colistin sulphate (10 µg), piperacillin 100 (µg) and Cefpodoxime Combination Disc Kit (Oxoid). The inhibition zones were measured and scored as sensitive, intermediate susceptibility and resistant according to CLSI documents (CLSI, 2014).

4.3 Results

A wide range of bacteria and parasites were detected in both *P. sicula* and *P. sicula klemmerii* individuals. Among the 24 analyzed animals, 23 (95.9%) were positive to at least one bacterium and 19 (79.1%) were positive to at least one parasite.
Regarding microbiological analysis, *Pantoea* spp. was isolated in 4/24 (16.7%) oral swabs, *Citrobacter* spp. in 1/24 (4.2%), *Morganella morganii* in 1/24 (4.2%), *Pseudomonas aeruginosa* in 1/24 (4.2%) and Coagulase-Negative Staphylococci (NCS) in 7/24 (29.1%). For cloacal swabs, *Citrobacter* spp. was found in 10/24 (41.7%) animals of which 1/10 (10.0%) was identified as *Citrobacter koseri*, *Enterobacter* spp. was found in 8/24 (33.3%) animals of which 1/8 (12.5%) was identified as *Enterobacter aerogenes*, *Escherichia coli* was found in 3/24 (12.5%) animals of which 1/3 (33.3%) was serotyped as serogroup O 145, *Morganella morganii* was isolated in 2/24 (8.3%) individuals, *Shewanella* spp. in 1/24 (4.2%), *Providencia* spp. in 2/24 (8.3%), Coagulase-Negative Staphylococci (NCS) in 20/24 (83.3%) and *Pseudomonas* spp. in 10/24 (41.7%) individuals. Several bacteria were routinely isolated from the same animal.
With respect to antimicrobial susceptibility testing, *Citrobacter* spp. showed the highest resistance profile. Specifically, two out of ten strains of *Citrobacter* spp. were resistant to three or more drugs ("multidrug-resistant") of which, one strain was resistant to ampicillin, doxycycline and streptomycin and the other one was resistant to ampicillin, amoxicillin-clavulanic acid, doxycycline and nitrofurantoin. The remaining strains were resistant to ampicillin, one was also resistant to doxycycline and one other was also resistant to amoxicillin-clavulanic acid. *Escherichia coli* O145 and all the strains of *Enterobacter* spp. were resistant to ampicillin. In contrast, *Pseudomonas aeruginosa* were susceptible to all antimicrobials tested.
Regarding the parasitological results, the scotch test highlighted the presence of *Ophionyssus natricis* mite. During the faecal examination, eggs of pinworms (20/24; 83.3%), *O. natricis* (12/24; 50%), and Dicrocoelidae (6/24; 25%) as well as oocysts of coccidia (11/24; 45.8%) were detected. As with bacteria, different parasite species were simultaneously detected from the same animal. In addition, adult liver flukes (Dicrocoelidae) were also found during the necropsy. The parasitological and microbiological results related to each lizard are detailed in Table 4.1.

Table 4.1 Parasites and bacteria found in Podarcis sicula klemmeri (LIC) and P. sicula (PLIC).

Lizard ID	Parasites				Bacteria	
		*EPG/OPG			Swab Samples	
	Parasite	NaCl	$ZnSO_4$	*O. natricis*	Cloacal	Oral
LIC 1001	Negative	–	–	Positive	*Enterobacter spp., Staphylococcus spp., Pseudomonas spp.*	*Pantoea* spp., *Staphylococcus* spp.
LIC 1002	Pinworms Coccidae	300 405	225 270	Positive	*Enterobacter aerogenes, Citrobacter* spp., *Staphylococcus* spp.	*Staphylococcus* spp.
LIC 1003	Pinworms Coccidae	720 120	150	Positive	*Morganella morganii, Citrobacter* spp., *Staphylococcus* spp.	*Pantoea* spp.
LIC 1004	Pinworms Coccidae	720 120	150	Positive	*Citrobacter* spp., *Shewanella* spp., *E. coli, Staphylococcus* spp., *Pseudomonas* spp.	*Pantoea* spp., *Staphylococcus* spp.
LIC 1005	Pinworms Liver flukes	2.880	1.440 960	Positive	*Citrobacter* spp., *Staphylococcus* spp.	*Staphylococcus* spp.
LIC 1006	Pinworms Liver flukes	2.880 120	1.440 120	Positive	*Morganella morganii, Citrobacter* spp., *Pseudomonas* spp.	*Morganella morganii, Citrobacter* spp., *Pantoea* spp. *Pantoea,PStaphylococcus*
LIC 1007	Pinworms Coccidae	3.600 4.140	2.100 1.530	Positive	*Enterobacter* spp., *E. coli, Staphylococcus* spp.	–
LIC 1008	Negative	–	–	Positive	*Enterobacter* spp., *Providencia* spp., *Staphylococcus* spp.	–
LIC 1009	Pinworms Coccidae	3.600 4.140	2.100 1.530	Positive	*E. coli* O145, *Staphylococcus* spp.	–
LIC 1010	Pinworms	330	300	Positive	*Staphylococcus* spp.	–
LIC 1011	Pinworms Liver flukes	3.420	1.890 120	Positive	*Pseudomonas* spp.	–
LIC 1012	Pinworms	3.420	1.890	Positive	*Citrobacter* spp., *Providencia* spp., *Staphylococcus* spp., *Pseudomonas* spp.	*Pseudomonas aeruginosa, Staphylococcus* spp.
LIC 1013	Pinworms Coccidae	870 360	690 105	–	*Enterobacter* spp., *Staphylococcus* spp.	–
LIC 1014	Pinworms Coccidae	870 360	690 105	–	*Citrobacter koseri, Staphylococcus* spp., *Pseudomonas* spp.	–
LIC 1015	Pinworms Liver flukes	540	300 7800	–	*Enterobacter* spp., *Staphylococcus* spp.	–
LIC 1016	Pinworms Liver flukes	540	300 42	–	*Staphylococcus* spp., *Pseudomonas* spp.	–
LIC 1017	Pinworms	540	300	–	*Pseudomonas* spp.	–
LIC 1018	Pinworms Coccidae	360 90	450	–	*Staphylococcus* spp.	–
LIC 1019	Pinworms Coccidae Liver flukes	360 99 90	450 9 48	–	*Staphylococcus* spp., *Pseudomonas* spp.	–
LIC 1020	Pinworms Coccidae	360 90	450 48	–	*Enterobacter* spp., *Staphylococcus* spp.	–
PLIC 1001	Pinworms	420	375	–	Not identified	*Staphylococcus* spp.
PLIC 1002	Negative	–	–	–	*Enterobacter* spp., *Citrobacter* spp., *Staphylococcus* spp.	–
PLIC 1003	Negative	–	–	–	*Citrobacter* spp., *Staphylococcus* spp.	*Staphylococcus* spp.
PLIC 1004	Negative	–	–	–	*Citrobacter* spp., *Staphylococcus* spp., *Pseudomonas* spp.	–

*EPG/OPG = eggs/oocysts per gram of faeces

4.4 Discussion

Our results provide new epidemiological data on bacterial and parasitic infections in *P. sicula* and *P. sicula klemmerii*. These species were poorly investigated from a sanitary perspective so far. Bacterial and parasitic infections in reptiles have recently gained scientific relevance. However, the majority of studies were carried out on captive-bred individuals of Cryptodira (Dipineto et al., 2012), Serpentes and Sauria (snakes and other 'lizards', (Rinaldi et al., 2012)). Conversely, data on infections in wild lizards are scarce.

The results of our study showed the presence of endoparasites (coccidia, pinworms and liver flukes) and ectoparasites (*O. natricis*) in *P. sicula* and *P. sicula klemmeri*. With respect to the presence of pinworms, our findings are in line with those by Casanova et al. (2003) who detected the presence of a nematode belonging to the Oxyuroidea superfamily in *P. sicula*. Nevertheless, our results showed the first epidemiological information on parasites infecting *P. sicula klemmeri*. Regarding the presence of *O. natricis*, there are no studies in literature that highlight its presence in *P. sicula* and *P. sicula klemmeri*, although the infestation by this mite has been described in various genus of Sauria as *Lacerta*, *Podarcis* and *Darevskia*. In addition, the carrier role of *O. natricis* in the transmission of a blood parasite belonging to the genus *Karyolysus* has been reported in lizards (Labbè et al., 1984). An interesting finding of our study was the detection at necropsy of liver flukes (Dicrocoelidae) in the continental lizards, although species identification was not performed.

Bacteriological results also added new epidemiological data in *P. sicula* due to detection of some potential zoonotic species as P. aeruginosa and *E. coli* O145. The bacterial isolation performed on oral swabs gave as result the presence of bacteria belonging to the genera *Pantoea*, *Pseudomonas*, *Morganella*, *Staphylococcus*, *Citrobacter*, *Shewanella* never detected before in *P. sicula* and *P. sicula klemmeri*. However, these bacteria are frequent in reptiles, along with other bacteria such as *Enterobacter* spp., *E. coli* and *Providencia* spp. (Jacobson, 1984; Joyner et al., 2006; Mayer and Frank, 1974). Bacteria isolated from cloacal swabs were *Enterobacter* spp., *Citrobacter* spp., *Morganella* spp., *E. coli*, *Providencia* spp. *Shewanella* spp., and *Pseudomonas* spp., gram-negative bacteria frequently isolated in other reptiles (Foti et al., 2009) as well as *Staphylococcus* spp. The isolation of *E. coli* O145 in one *P. sicula klemmeri* is noteworthy due to the potential zoonotic role of this serogroup which is considered a shigatoxin-producing *E. coli*.

It is interesting to notice that the insular population presents similar bacterial diversity of its mainland counterpart, although the differences in sample size urge caution in interpreting these results. Noteworthy, 9 different bacterial genera were identified in *P. s. klemmeri* (up to six in a single individual), against 4 in mainland individuals (up to three in a single individual). A weakness of this study was the failure to isolate bacteria in the oral cavity of some lizards due to the difficult to keep viable some strains during the isolation procedures. To our knowledge this is the first study to assess the antimicrobial resistance profiles of potentially zoonotic bacteria carried by *Podarcis* spp. It is difficult to speculate regarding the results of the antimicrobial resistance recovered in the present study. However, one possible mechanism by which lizards acquire antimicrobial resistant bacteria in their environment range may be directly through exposure to human or livestock waste, or indirectly through consumption of prey which may harbour resistant bacteria. The figure for parasites is even harder to interpret given the smaller sample size. Yet, we found coccidia, liver flukes and pinworms in the insular populations, and pinworms only (in just one individual) within continental lizards. While difference in collecting season, sample size, and the instance of necropsy in two individuals only suggest great caution, data seem to indicate an overall higher parasite/bacterial load in insular lizards. Assuming this is true, it remains to be elucidated whether this depends on population density on Licosa, or on less competent immune system in insular melanic individuals (Raia et al., 2010; Raia et al., 2010a).

In conclusion, the results of this study highlighted various bacteria and parasites, some of them pathogenic, able to infect the species *P. sicula* and the subspecies *P. sicula klemmeri*. Further studies

are needed to better understand the epidemiology and transmission routes of these pathogens along with their impact on the welfare and behaviour of Italian wall lizards.

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Chapter 5

Salmonella serotypes isolated in geckos kept in seven collections in southern Italy*

*Based on the manuscript: Russo TP, **Varriale L**, Borrelli L, Pace A, Latronico M, Menna LF, Fioretti A, Dipineto L, 2018. *Salmonella* serotypes isolated in geckos kept in seven collections in southern Italy. J Small Anim Pract 59, 294-297.

5.1 Introduction

Salmonella species are natural inhabitants of the reptile gut microflora, detected in various species of reptiles kept as pets (Dipineto *et al*. 2012, Chen *et al*. 2013, Mughini-Gras *et al*. 2016). Clinical signs of *Salmonella* infection in reptiles are rare and appear to be associated with underlying disease or other stressors. A causal relationship between *Salmonella* infection and disease is, indeed, generally difficult to establish (Hoelzer *et al*. 2011). Reptile-associated *Salmonella* infections in humans are increasing in recent years probably because they are increasingly being kept as pets (Mughini-Gras *et al*. 2016). Bosch *et al*. (2016) reported that exposure to small turtles has been recognised as a source of human salmonellosis in the USA since the 1960s, when small baby turtles first became popular pets. Furthermore, two case–control studies of human salmonellosis, performed to estimate the burden of reptile- and amphibian-associated *Salmonella* infections, suggested that reptile and amphibian exposure is associated with approximately 74,000 *Salmonella* infections annually in the USA (Mermin *et al*. 2004). Cases of *Salmonella* infection attributed to direct or indirect contact with reptiles or other exotic pets have also been described in a number of European countries, but a more comprehensive overview of the magnitude of this problem in Europe is lacking (Editorial Team *et al*. 2008). Geckos are often reported as carriers of many zoonotic enteropathogens, including non-typhoidal salmonellae (Singh *et al*. 2013). This study aimed to evaluate the prevalence of *Salmonella* species in captive geckos in southern Italy.

5.2 Materials and Methods

5.2.1 Sampling

During the period April to July 2016, faecal swabs were collected from 70 clinically healthy captive geckos belonging to four species, as indicated in Table 1: *Correlophus ciliatus* (n=36), *Gekko vittatus* (n=16), *Ptychozoon kuhli* (n=8) and *Phelsuma madagascariensis grandis* (n=10).
All the geckos examined were selected from seven private owners located in the provinces of Napoli and Salerno (southern Italy). Each owner lived in an urban area and bred around eight to 12 geckos, separated by species and kept in pairs in each terrarium. Faecal swabs were collected taking care that they did not come into human or animal contact before, during or after collection of samples. To collect samples, sterile masks and gloves were worn, and gloves were changed after every sample. Before the collection of faecal samples, a sheet of sterile aluminium foil was placed under the grid of each cage overnight. Samples were then collected by sterile cotton-tipped swabs. Each sample swab was inoculated in Buffered Peptone Water (Oxoid), stored at +4°C, transported to the laboratory and analysed within 2 hours of collection. Reptile-handling procedures were performed with informed consent of the owners.

5.2.2 Bacterial Isolation

All samples were analysed for isolation of *Salmonella*. Samples were incubated at 37°C for 18 hours. After incubation, samples were inoculated into Rappaport–Vassiliadis Broth (Oxoid) and incubated at 42°C for 18 hours. Cultures obtained were plated onto Xylose-Lysine-Desoxycholate Agar (Oxoid), incubated at 37°C and examined after 24 hours. Suspected *Salmonella* colonies were inoculated into a second selective media, Brilliance *Salmonella* Agar (Oxoid), and incubated at 37°C for 24 hours. All isolates were biochemically identified using the API20-E system (bioMérieux) and Triple Sugar Iron Agar (Oxoid). All strains were stored frozen at −80°C in 20% glycerol until serotyping was performed. Salmonella isolates were serotyped according to the Kauffman-White scheme. Analyses were carried out in collaboration with the National Reference Laboratory for *Salmonella* (IZSVe). Prevalence and confidence intervals were calculated using a Microsoft Excel spreadsheet.

5.2.3 Antimicrobial Susceptibility Testing

All isolates were submitted for antimicrobial susceptibility testing using the disc diffusion method according to Clinical Laboratory Standards Institute (CLSI 2012). The antimicrobials tested were amoxicillin (10 μg), ampicillin (10 μm), ceftazidime (30 μg), ciprofloxacin (5 μg), enrofloxacin (5 μg), sulfamethoxazole-trimethoprim (23.75/1.25 μg) and tetracycline (30 μg). *Salmonella* typhimurium ATCC 14028 was used as a control strain in each experiment. The inhibition zones were measured and scored as sensitive, intermediate susceptibility and resistant according to the CLSI documents (CLSI 2014).

5.3 Results

Salmonella species were isolated from 24 of 70 (34.3%; 95% confidence interval 23.6 to 43.7%) geckos examined. Specifically, *Salmonella* species was isolated from 22 of 36 faecal swabs collected from *Correlophus ciliatus* and from two of eight faecal swabs collected from *Ptychozoon kuhli*. In contrast, *Gekko vittatus* and *Phelsuma madagascariensis grandis* were consistently negative. Of the 24 *Salmonella isolates*, 41.7% (10/24) were serotyped as *Salmonella* Muenchen, 16.7% (4/24) as *Salmonella* Hadar, 16.7% (4/24) as *Salmonella* Oranienburg, 8.3% (2/24) as *Salmonella* Eastbourne, 8.3% (2/24) as Salmonella enterica subspecies *diarizonae* 61:r:z53 and 8.3% (2/24) as *S. enterica* subspecies *houtenae* 43:z4, z23:-. Results are summarised in Tables 5.1 and 5.2.
In antimicrobial susceptibility testing, no strain was resistant to three or more drugs (i.e., not "multidrug-resistant"). All the strains of *S.* Muenchen, *S.* Hadar and *S.* Eastbourne were resistant to ceftazidime; S. enterica subspecies *diarizonae* was resistant to ampicillin. Finally, two of the four strains of *S.* Oranienburg were resistant to ceftazidime and the other two to ampicillin. In contrast, the other strains isolated were susceptible to all antimicrobials tested.

Table 5.1 Prevalence of Salmonella species in different species of geckos bred in southern Italy.

Species	Number examined	*Salmonella* species Number positives	%	95% Confidence Interval
Correlophus Ciliatus	36	22	61.1%	43.5-76.4
Gekko vittatus	16	0	-	-
Ptychozoon kuhli	8	2	25%	4.5-64.4
Phelsuma madagascariensis grandis	10	0	-	-
Total	70	24	34.3%	23.6-46.7

Table 5.2 Salmonella serotypes identified in Correlophus ciliatus and Ptychozoon kuhli.

Species	N. of positive / N. of geckos examined	Serotypes of *Salmonella* species	N. of *Salmonella* serotypes/ N. of positive
Correlophus ciliatus	22/36	*S.* Muenchen	10/22
		S. Hadar	4/22
		S. Oranienburg	4/22
		S. Eastbourne	2/22
		S. enterica subsp. *diarizonae*	2/22
Ptychozoon kuhli	2/8	*S. enterica* subsp. *houtenae*	2/2

5.4 Discussion

Salmonella species occurs naturally in the gastrointestinal tract of many reptiles; it is commonly shed by these animals, and around the world, a large number of serotypes have been isolated from feral and captive reptiles as well as from their eggs. It has been estimated that as many as 90% of all captive reptiles carry *Salmonella*, including a large number of "reptile-associated" as well as "broad host-range" serotypes (Hoelzer et al. 2011).
The results of the present study identified *Salmonella* species in geckos kept as pets in southern Italy, with a prevalence of 34.3%. Previous studies on the prevalence of Salmonella species in geckos performed worldwide are scant and refer to different and various species of this reptile but show heterogeneous results. In a recent study conducted by Jiménez et al. (2015) *Salmonella* species were isolated from 4.3% samples collected from 115 common house geckos (Hemidactylus frenatus) in Costa Rica. In a study conducted by Singh et al. (2013) *Salmonella* species were isolated from 8.8% samples collected from 194 common house geckos in India. Finally, in a survey conducted by Smith et al. (2012), *Salmonella* species were isolated from 80% wild-caught tokay geckos (*Gekko gecko*) imported to the USA from Indonesia. Our results are not in line with these previous studies, and this may be influenced by their living conditions (captivity versus free-living), species and geographical circumstances. The most prevalent *Salmonella* serotypes isolated in our study were *S.* Muenchen, *S.* Hadar and *S.* Oranienburg. Data available in literature about these *Salmonella* serotypes in geckos are fragmentary. The Centers of Disease Control and Prevention (CDC) announced an outbreak of *S.* Muenchen in humans linked to contact with pet crested geckos (*Correlophus ciliatus*) purchased from multiple pet stores in different states of the USA (CDC 2015), whereas *S.* Hadar, which is a zoonotic poultry-associated serotype, was mentioned in a Serbian report (Bošnjak et al. 2016) from a Leopard gecko (*Eublepharis macularius*). With respect to *S.* Oranienburg, this is believed to be the first report from a gecko, although this *Salmonella* species has been isolated from other reptile species, including anoles (*Sumiyama* et al. 2014), iguanas (*Sylvester* et al. 2014) and snakes (Dipineto et al. 2014). *S. enterica* subspecies *diarizonae* is consistently isolated from reptiles (Franco et al. 2011, Dipineto et al. 2014, Wikström et al. 2014) and anecdotally from geckos (Bošnjak et al. 2016). In contrast, *S.* Eastbourne and *S. enterica* subspecies *houtenae* have been frequently reported in geckos (Sadek & Refai 1968, Singh et al. 2013, Le Souëf et al. 2015). It is noteworthy that all the serotypes isolated in the present study have been associated with outbreaks of human salmonellosis in several countries (Mitchell-Jones & Sankey 1984, Nimir et al. 2011, Hervás et al. 2012).
Worldwide, the prevalence of antibiotic resistance in *Salmonella* species has increased, which is becoming a serious problem for treatment (Chen et al. 2013). In the present study, 18 *Salmonella* isolates (75%) expressed resistance to ceftazidime. This contrasts with a survey conducted on *Hemidactylus frenatus* by Jiménez et al. (2015), who showed a high susceptibility of *Salmonella* species to this antimicrobial. Instead, four additional strains isolated in the present study expressed resistance to ampicillin (17%), in line with previous studies conducted on other species of gecko (Smith et al. 2012, Singh et al. 2013). Data about the antimicrobial susceptibility of *S.* Oranienburg in geckos are not available, so we cannot compare our results with other studies. However, we reported that two *S.* Oranienburg strains were resistant to ampicillin and the other two to ceftazidime. Our results demonstrate that geckos may play a role in the epidemiology of human salmonellosis. Prime examples are the two clinical cases described by Sanyal et al. (1997), who reported two children with signs and symptoms of gastroenteritis in the UK. *S. chameleon* was isolated from the stool of one child and also from an iguana kept in the home as a pet. *S. arizonae* was isolated from the stool of the other child and also from four snakes sharing the same household. In light of these findings, the authors of the study discouraged the ownership of reptiles in households with children younger than 5 years of age. Clearly, more detailed epidemiological studies are needed to better understand the role of pet geckos within human salmonellosis as well as to accurately quantify the risk. In the present study, it was not possible to speculate about the source of *Salmonella* in geckos. In fact, as also reported by Sanyal et al. (1997), reptiles are symptomless carriers of *Salmonella* species,

probably becoming infected through contaminated food, water or soil, and the fact that the animals show no symptoms may indicate that the bacteria exist as commensal flora in the animal's gut. The higher prevalence of certain serotypes could be a result of potential cross-infection between animals, which could occur during housing on the farms or exotic animal fairs, where these reptiles are purchased. In conclusion, until more is known about the epidemiology and prevention of these infections in geckos, caution should be exercised in translocation, husbandry and human contact with these animals.

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Chapter 6

Microbiological survey of sugar gliders (*Petaurus breviceps*) kept as pets in Italy*

*Based on the manuscript: **Varriale L**, Russo TP, Pace A, Mediatore S, Borrelli, Santaniello A, Menna LF, Fioretti A, Dipineto L, 2019. Microbiological survey of sugar gliders (Petaurus breviceps) kept as pets in Italy. Lett Appl Microbiol 69, 399-402.

6.1 Introduction

Sugar gliders (*Petaurus breviceps*) are small tree-dwelling mammals that are one of the most commonly traded exotic pets in the United States (Nichols et al., 2015). The current size of the USA sugar glider population remains unknown, but it makes up a proportion of the 3_5 million exotic mammalian pets kept in private households (excluding ferrets, rabbits and livestock) (Campbell et al., 2019). In order to estimate the growth of the sugar gliders population in our country, the Italian sugar glider association (AIPDZ) created a national database online, in which breeders and private owners can register their animals and obtain information about the genealogy of the specimens kept. Currently, this database holds more than 2000 sugar glider records, but the number may be underestimated because not all animals are registered. In the literature, small exotic pet mammals, including sugar gliders, are mentioned as potential carriers of zoonotic pathogens (Woodward et al., 1997). Anderson et al. (2017) reported a multistate human salmonellosis outbreak linked to pet hedgehogs in the United States. Sugar gliders are susceptible to *Clostridium piliforme* and *Giardia* spp. infection, although no documented cases of zoonotic transmission from gliders to humans have been reported in the literature (Brust, 2009). Other cases of human infection with a firmly established epidemiological link to exotic pets were documented in Canada, such as five cases of human salmonellosis associated with sugar gliders (Woodward et al., 1997). However, few data are available on the role of sugar gliders as potential carriers of zoonotic bacteria and source of infection for human and other companion animals. This study was performed to determine the prevalence of potentially zoonotic bacteria (Salmonella spp., *Escherichia coli*, *Campylobacter* spp., *Pseudomonas* spp., *Klebsiella* spp., *Listeria monocytogenes* and *Yersinia enterocolitica*) in 64 sugar gliders kept as pets in different areas of Italy.

6.2 Materials and Methods

6.2.1 Sampling

During the period January 2017/September 2018 rectal swabs were collected from 64 clinically healthy sugar gliders (*P. breviceps*) selected from private owners located in different areas of Italy. Each owner lived in an urban area and bred 2 sugar gliders kept in pair in each bird cage. For each animal a questionnaire was administrated including sex, age, origin, number of animals of the colony, diet and related method of conservation, any previous symptoms and pathologies, previous treatments and cohabitation with other animals. Of the 64 animals, 34 were young and 30 adults, of these 22 were males and 42 females. Rectal swabs were collected taking care that they did not come into human or animal contact before, during or after collection of samples. To collect samples sterile gloves were worn and sterile cotton-tipped swabs were used.
Each sample swab was inoculated in phosphate-buffered saline (PBS), stored at +4°C, transported to the laboratory and analyzed within 2 h of collection. Sugar glider-handling procedures were performed with informed consent of the owners.

6.2.2 Bacterial Isolation

All samples were analysed for isolation of Enterobacteriaceae (*Salmonella* spp., *Citrobacter* spp., *Enterobacter* spp., *Escherichia coli*, *Klebsiella* spp), *Campylobacter* spp., *Pseudomonas* spp., *Listeria monocytogenes* and *Yersinia enterocolitica*. Samples stored in PBS were inoculated in buffered peptone water (BPW; Oxoid, Milan, Italy), *Campylobacter* selective enrichment broth (CSEB; Oxoid), *Listeria* primary selective enrichment medium (UVM I; Oxoid) and *Listeria* secondary selective enrichment medium (UVM II; Oxoid) and incubated at 37°C for 24 h except for CSEB that were incubated in microaerophilic atmosphere at 42°C for 48 h. Subsequently samples were inoculated/streaked into Rappaport-Vassiliadis broth (RV; Oxoid), *Pseudomonas* cetrimide agar (PCA; Oxoid), MacConkey agar (MCA; Oxoid) and *Listeria* selective Agar Base (Oxford; Oxoid), chromogenic *Listeria* agar (OCLA; Oxoid) and *Campylobacter* blood-free selective agar (CBFA; Oxoid). The remaining PBS were incubated at 4°C for 14 days, streaked on *Yersinia* selective agar base (cefsulodin-irgasan-novobiocin, CIN Agar; Oxoid) and incubated at 30°C for additional 24–48 h. PCA, MCA, Oxford and OCLA were incubated at 37°C for 24–48 h, whereas RV was incubated at 42°C for 24–48 h and then streaked on xylose lysine desoxycholate agar (XLD; Oxoid) and brilliant green agar (BGA; Oxoid); CBFA were incubated in microaerophilic atmosphere at 42°C for 24–48 h. All the isolates were previously identified according to their morphological features, their growing, Gram staining, motility and pigments production test and through conventional biochemical and phenotypic test. Progressively, the isolates were identified through the biochemical systems API 20 E, API 20 NE (bioMerieux, Mercy-l'Etoile, France).
Prevalence and confidence intervals were calculated using a Microsoft Excel spreadsheet.

6.2.3 Antimicrobial Susceptibility Testing

All isolates were submitted for antimicrobial susceptibility testing using the disc diffusion method according to Clinical Laboratory Standards Institute (CLSI 2012). The antimicrobials tested were nalidixic acid (30 µg), ampicillin (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamicin (30 µg), streptomycin (10 µg), tetracycline (30 µg), enrofloxacin (5 µg), amoxicillin/clavulanic acid (30 µg), doxycycline (30 µg), colistin sulphate (10 µg) and Cefpodoxime Combination Disc Kit (Oxoid). The inhibition zones were measured and scored as sensitive, intermediate susceptibility and resistant according to CLSI documents (CLSI 2012, 2014) and The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019).

6.3 Results

A wide range of bacteria were detected in all the sugar gliders examined. The highest prevalence concerned members of the family Enterobacteriaceae, in particular *Citrobacter* spp. (50%), *Enterobacter* spp. (28.1%) and *Klebsiella pneumoniae* (15.6%), *Pseudomonas aeruginosa* was isolated in 10 animals out of 64 (15.6%). All the samples were consistently negative for Salmonella spp., *Campylobacter* spp., Listeria monocytogenes and *Yersinia enterocolitica*. Results are summarized in Table 6.1.
With respect to antimicrobial susceptibility testing, 10 strains of *Klebsiella pneumoniae* were resistant to three or more drugs (i.e., "multidrug-resistant"), in particular they showed resistance to amoxicillin, ampicillin, doxycycline and ceftazidime, whereas one of the two strains of *Klebsiella oxytoca* was resistant to amoxicillin/clavulanic acid and 10 strains of *Pseudomonas aeruginosa* showed resistance to ceftazidime. In contrast, the other strains isolated were susceptible to all antimicrobials tested.

Table 6.1 Prevalence and related 95% confidence interval of the bacteria isolated in sugar gliders.

Bacteria	N. positives/ N. examined	Prevalence %	95% Confidence Interval
Citrobacter spp.	32/64	50.0	32.4 – 62.7
Enterobacter spp.	18/64	28.1	17.9 – 40.9
Klebsiella pneumoniae	10/64	15.6	8.1 – 27.3
Pseudomnas aeruginosa	10/64	15.6	8.1 – 27.3
Klebsiella oxytoca	2/64	3.1	0.5 – 11.8
Escherichia coli	2/64	3.1	0.5 – 11.8
Serratia marcescens	2/64	3.1	0.5 – 11.8

6.4 Discussion

Bacteria potentially pathogenic to humans were found in all sugar gliders examined. The highest prevalence of infection pertained to members of the family Enterobacteriaceae. Those isolated occur commonly in the gastrointestinal tract of many warm- and cold-blooded species (Guentzel, 1996). *Enterobacter* spp., *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* represent emerging nosocomial pathogens that may cause urinary tract infections (Khatri et al., 2012), respiratory infections, septicaemia (Mezzatesta et al., 2012), and infections in susceptible patients such as children (Pereira et al., 2013) and people with weakened immune systems (Yadegarynia et al. 2013). *Citrobacter* spp. has been related to cases of peritonitis in peritoneal dialysis patients (Carlini et al., 2005; Chao et al., 2013; Chen et al., 2013), to cases of meningitis, and brain abscesses in newborns (Plakkal et al., 2013). The failure to isolate *Yersinia enterocolitica* concurs with the results of a previous study conducted in Japan by Kameyama et al. (2016), in which four sugar gliders examined from a pet store were negative for the detection of this bacterium. Scant information in the literature is available on the occurrence of *Salmonella* spp. in sugar gliders. However, Woodward et al. (1997) reported five cases of human salmonellosis caused by *Salmonella* Tilene with a firmly established epidemiological link to sugar gliders. The absence of *Campylobacter* spp. should be treated with caution. To our knowledge this bacterium has not been investigated previously in sugar gliders. In the present survey no strains of *Listeria monocytogenes* were detected. Nicholls et al. (2015) reported on a case of listeriosis causing sepsis in a sugar glider in New Mexico, the animal consuming human food items, including cantaloupes. Concurrently, a national outbreak of *L. monocytogenes* foodborne illness associated with cantaloupes in humans occurred, although the bacterial strains were genetically distinct. The ability of foodborne pathogens to infect exotic pets is poorly investigated (Russo et al., 2018) and, although rare, should be considered, especially for sugar gliders whose diet consists of many exotic fruits often imported from other countries and subjected to handling and storage that could negatively affect their microbiological quality. Also, insects consumed in the sugar gliders' diet may carry pathogens even at long distances from their origin and breeding sites. To avoid bacterial infections and anthropozoonotic transmission, uneaten foods should be removed from cages promptly and all the items should be washed prior to administration.

Moreover, it is recommended that owners and veterinarians wash their hands both before and after handling sugar gliders, with particular caution for pregnant women, children, elderly and all people with weakened immune systems (Swaminathan and Gerner-Smidt 2007).

To our knowledge this is the first study to assess the antimicrobial resistance profiles of potentially zoonotic bacteria carried by *P. breviceps*. It is difficult to speculate regarding the results of the antimicrobial resistance recovered in this study. However, one possible mechanism by which sugar gliders acquire antimicrobial resistant bacteria may occur directly through exposure to human and other animals or indirectly through consumption of insects which may harbor resistant bacteria.

In conclusion, the results of this study highlighted various bacteria, some of them potentially pathogenic for other animal species and humans, able to infect *P. breviceps*. Further studies are needed to establish the epidemiological role that sugar gliders may act in the spread and transmission of these agents as well as to accurately quantify the risk. Finally, since sugar gliders are becoming popular as pets worldwide, until more is known about the epidemiology and prevention of infectious diseases in these animals, caution should be exercised in husbandry and human contact with them.

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Chapter 7

Discussion

The “One Health” approach to tackle the Antimicrobial Resistance: present assessments and future perspectives

7.1 Introduction

Antibiotic resistance is a multifaceted ecosystem problem that threatens the interdependent humans, animals and environmental health linked together under the "One Health" framework (Iskandar et al., 2020). Despite the increased dissemination in the environment of clinically relevant ARGs released from anthropogenic sources, it is still difficult to predict and fully understand the evolution and ecology of antibiotic resistance in this setting (Berendonk et al., 2015).
The present thesis provided preliminary results on the role of wildlife as reservoir and potential source of AMR, although the studies reported reflect the general lack of an established link with the spread of these resistant determinants within the human-animal-environment interface.
In particular, significant findings emerged regarding: i) the detection of phenotypic resistance patterns of *Campylobacter* spp. from Mediterranean seagulls towards macrolides that usually represent the first line of defense in human Campylobcateriosis (Chapter 2); ii) the emergence in companion birds of MDR strains of *E. coli* and *Pseudomonas aeruginosa* harbouring resistant determinants potentially transmissible directly or indirectly to humans (Chapter 3); iii) the first report of some potential zoonotic species as *P. aeruginosa* and *E. coli* O145 from an insular population of Italian lizards (*Podarcis sicula klemmeri*), with the latter strain displaying a resistance pattern towards ampicillin (Chapter 4); iv) the potential role of reptiles kept as pets (geckos from Italian breedings) in the spread of resistant serotypes of *Salmonella* spp. previously associated with outbreaks of human Salmonellosis worldwide (Chapter 5); v) the first assessment of the antimicrobial resistance profiles of potentially zoonotic bacteria carried by exotic marsupials bred as pets (i.e., *Petaurus breviceps*), such as *P. aeruginosa* and MDR *Klebsiella pneumoniae* (Chapter 6).
The main limitation of the aforementioned studies is the lack of a molecular characterization of the resistance phenotypes that may explain the mechanisms behind the acquisition of these determinants, as well as the dispersion in the environment and the potential risks related to the close contact between humans and animal species other than livestock. On the other hand, the most important objective of this thesis was to emphasize the need to address this topic from a "One Health" perspective, encouraging further and more in-depth research to fill the current gaps in this field and effectively quantify the risks for the ecosystem health. Therefore, in the next paragraphs will be discussed the importance of a "One Health" approach to antimicrobial resistance surveillance, prevention and control, as well as the present assessments and future perspectives regarding the novel therapeutical strategies as alternatives to antibiotics.

7.2 The evolution of "One Health" concept and its application in the integrated approach to AMR containment

The "One Health" concept finds its origins with the veterinary epidemiologist Calvin Schwabe, who introduced the new formula "One Medicine" as an integrated approach between human and animal medicine to combat zoonoses (Schwabe, 1984). In the early 2000s this concept also began to encompass the field of environment, in response to the increased health threats including global warming, food insecurity and the emergence of new infectious diseases. In 2004, the non-governmental association Wildlife Conservation Society organized the first international conference "One World, One Health: Building Interdisciplinary Bridges to Health in a Globalized World" renaming the old formula "One Medicine" and leading to the publication of a preliminary international guide, "The Manhattan Principles" (Wildlife Conservation Society, 2004).
Lastly, in 2010, the WHO, OIE and FAO published a new agreement which redefined the "One Health" concept, also including the food sector: "A world capable of preventing, detecting, containing, eliminating, and responding to animal and public health risks attributable to zoonoses and animal diseases with an impact on food security through multi-sectorial cooperation and strong partnerships" (Badau, 2021).

The adoption of the "One Health" approach as a solution to the antibiotic resistance problem dated 2014, with the WHO report on microbial resistance surveillance produced in collaboration with OIE and FAO (WHO, 2014), including a definition of the "One Health" concept that covers human and animal health, as well as food safety. The report was followed in 2015 by the launch of the first global plan (GAP) by the WHO, OIE and FAO (WHO, 2015), in order to develop solutions and measures to control health risks attributable to AMR. Five years on, 117 countries have adapted the GAP to produce their own national action plan (NAP), yet many countries still face growing levels of antimicrobial consumption and AMR rates. Surveillance, infection prevention, responsible use, universal access and research development have been identified as the main policy goals to tackle AMR (Ogyu et al., 2020).
"One Health" promotes a paradigm shift from the current individual- or disease-centered approach to a more integrated "systems" or "community-based" concept, that includes how we manage the environments we share. Changes in the interdependent relationships of humans, animals and ecosystems can alter the health of all three and also influence the way human and animal populations co-exist. What the "One Health" approach offers is an even broader multi-systems perspective on what health means and the inclusion of a wider range of expertise to include areas of academic specialization such as veterinary as well as human medicine, ecology and environmental management, agriculture, social sciences and engineering (Conrad et al., 2013).

7.2.1 Antimicrobial consumption and antimicrobial resistance surveillance

Despite the WHO calling for local, national and global AMR surveillance systems to be established, gaps in surveillance remain, primarily through lack of capacity and integration. The US National Action Plan proposed the strengthening of a "One Health" national surveillance system (for humans, animals and the environment) with improved international collaboration and capacity (Queenan et al., 2016). Antimicrobial administration and the consequent selective pressure have been recognized as the main drivers for AMR (Shallcross et al., 2014). Therefore, it is fundamental to link antimicrobial consumption data with AMR surveillance data in order to define methods for AMR containment. The combined surveillance would also facilitate monitoring of the impact of interventions aimed to improve antibiotic stewardship and reduce consumption (Aryee and Price, 2014). Consumption data in humans are collected in hospital and community settings, while in animals they are gathered at the species level, such as food-producing animals (including fish), companion animals and wildlife. Data interpretation requires an integrated analysis by sectoral experts, as well as a centralized program to set standards for data collection. Despite the growing evidence that the evolution and spread of AMR in the environment contributes to the occurrence of AMR in human and urban settings, standardized methods that are directly applicable to environmental samples (which allow reliable comparisons with clinical data) have never been developed. Adapting clinical criteria to environmental samples (soil, water, sludge, manure, sediment) represents a drawback since most environmental bacteria are not recovered in culture-dependent surveys and further, the definitions of resistance used for clinical isolates, which are mainly based on the possible therapeutic failure of human or animal bacterial infections, may not apply to environmental bacteria (Berendonk et al., 2015). Culture-independent approaches (e.g., quantitative PCR) can give an approximation of the presence of known ARGs in environmental samples, although harmonized guidelines which allow direct comparisons between different environmental settings and bridges with clinical data, are needed. In order to obtain a global perspective of environmental resistome, some bacterial species have been suggested as indicators (i.e., *E. coli*, faecal Enterococci, *P. aerugionosa*, A*eromonas* spp. and *K. pneumoniae*) along with the most frequently genes occurring in environmental compartments that are subjected to human activities (Vaz-Moreira et al., 2014). Currently, one of the major challenges is the consolidation of specialized databases to integrate information from routine monitoring and data collected by research studies aimed to understand the acquisition and molecular evolution processes. With this in mind, databases focusing on environmental antibiotic

resistance and the comparisons with the existing databases on antibiotic resistant bacteria and ARGs in clinical, veterinary and food-associated products, may elucidate the possible implications in human and animal health (Berendonk et al., 2015). The improvements in modern technologies, such as whole-genome, functional metagenomic and transcriptome analyses will be essential to clarify the interaction between the environmental and genomic context, and to understand how this relationship will influence the selection of specific ARGs, as well as to reveal unexpected hotspots of antibiotic resistance (Nesme et al., 2014). In this context, two databases, the Antibiotic Resistance Database (ARDB) and the Comprehensive Antibiotic Resistance Database (CARD) assembled in the last decade are expected to provide computational tools for the rapid prediction of antibiotic resistance genes and their targets in newly sequenced genomes and establish phylogenetic relationships (Peterson and Kaur, 2018).

7.2.2 Other recommended steps in managing the antimicrobial resistance threat

The adoption of antibiotics stewardship programs or their implementation by interdisciplinary teams and educational interventions has been shown to obtain significant reduction in antibiotic consumption, duration and inappropriate use (Barlett et al., 2013). Ideally, antimicrobial stewardship should be designed including the following: (a) passive educational measures (antibiotic guidelines, educational sessions), (b) active interventions (clinical rounds, prospective audits, reassessment of antibiotic perspectives), (c) restrictive measures (limiting antibiotics on the hospital formulary, reporting of susceptibility by the microbiology laboratory, antibiotic order form, preauthorization), and (d) supplemental measures (computer-assisted management programs, multidisciplinary stewardship teams, consultancy services) (Roca et al., 2015). Despite the evidence that a shorter course of antibiotic administration may be just as effective, most guidelines still recommend relatively prolonged or imprecise treatment durations, thus facilitating the selective pressure on bacterial species and the resultant development of resistance. (Luyt et al., 2014). Human behaviour and attitudes regarding antibiotic prescribing and use should be considered in both human and veterinary medicine. Decisions around antimicrobial usage in livestock should consider the trade-offs that occur between improving animal health, productivity and animal welfare, as well as impacts on hunger and poverty decreasing versus the risk of driving resistance (Rushton, 2015). The ongoing nature of the AMR crisis will therefore require a shift in the general perception of antimicrobials and their use at all levels of society, minimizing their use whenever possible and preventing the elimination products from entering the wider environment (Michael et al., 2014). The recommendations and the resultant actions and programs promoted by government and international initiatives focus on four main action areas: i) disease prevention through the use of vector control, vaccination, public education, clinical education, and legislative action; ii) tracing of resistant bacteria; iii) improvement of antibiotic use; iv) rapid and effective disease management through the use of new diagnostic tools and development of new antibiotics (Ventola, 2015). Although so far these initiatives have been useful in the management of AMR containment, the increasing incidence of infections resistant to antimicrobial therapy indicates that these measures should be expanded and implemented if the reduction of resistant microbial infections must be achieved.

7.2.3 Novel antimicrobial drugs and antibiotics in combination

Despite the discovery and development of new antimicrobial drugs should be a global priority, many pharmaceutical industries have left antibiotic drug discovery programs due to the difficulty in predicting the development of resistance and the complex and divergent regulatory requirements, although some initiatives have been suggested to encourage investment by the pharmaceutical companies (Roca et al., 2015). In the decade between 2000 and 2010 only five new antibiotics have been approved for clinical use, whereas four new antibiotics were approved in 2014 and only one in 2015 (Ventola, 2015). With respect to ESKAPE pathogens, every passing year the overall number of

effective antibiotics is declining, which is predisposing us toward a future with antibiotics that are ineffective. Moreover, many antibiotics suggested against ESKAPE since 2010 have been deleted from the antibiotic lists recommended in the CLSI guidelines, with addition of relatively few antibiotics/antibiotic combinations (Mulani et al., 2019). The epidemiological dimension of increased resistance to β-lactam antibiotics (BLAs) is mainly linked with the global spread of plasmid-mediated β-lactamases that threaten the clinical effectiveness of all current BLAs, however a promising strategy has been the development of β-lactamase inhibitors (BLIs) capable of restoring the activity of β-lactam drugs or alternatively to develop new compounds from this class (Vrancianu et al., 2020). Since the discovery of clavulanic acid as an inhibitor of most class A β-lactamases, various combinations of penicillins and inhibitors (amoxicillin-clavulanate, ampicillin-sulbactam, piperacillin-tazobactam) have been used to treat infections caused by β-lactamase-producing pathogens (White et al., 2004). Among them, one of the most relevant categories of recently introduction is diazabicyclooctanones (DBOs), with avibactam being the first inhibitor successfully used in the clinic in combination with ceftazidime and clinically approved for treating abdominal and urinary tract infections (UTIs) and pneumonia (Zhanel et al., 2013; Tuon et al., 2018). For the next generation of combined therapy, other BLIs have been approved for clinical use such as the diazabicyclooctanes (DABCOs) (avibactam, relebactam, macubactam, zidebactam, and nacubactam), which have been shown to augment the activity of β-lactams in the absence of β-lactamases in different species of MDR Gram-negative bacteria (Thomson et al., 2019). Clinical trials have revealed that some of the most potent formulations in the fight against MDR carbapenemase producing Enterobacteriaceae (CRE) are Ceftazidime-Avibactam (CAZ-AVI), Imipenem/Relebactam (IMI-REL) and Meropenem/Vaborbactam (MEM-VAB) (Vrancianu et al., 2020). CAZ-AVI is an intravenous combination recommended for treating complicated intraabdominal infections in combination with metronidazole, pyelonephritis and other UTIs, pneumonia and other critical disease caused by Gram-negative pathogens, bringing significant advantages over many non-susceptible ceftazidime species, such as some Enterobacteriaceae and *P. aeruginosa*, while its activity on *Acinetobacter* spp., Gram-positive cocci, and anaerobes remains moderate (Shields et al., 2017). The IMI-REL activity has been evaluated *in vitro* showing susceptibility for 94% of *P. aeruginosa* isolates, while have been reported variable susceptibility levels in carbapenem-resistant Enterobacteriaceae (from minimum level to 100% susceptibility in *K. pneumoniae* KPC-2 and KPC-3 producing isolates) and low in *Acinetobacter baumannii* tested isolates (Lapuebla et al., 2015; Lob et al., 2017; Canver et al., 2019). MER-VAB has been reported as potent combination *in vitro* against nosocomial *E. coli* isolates co-producing AmpC and KPC, and the restored activity of MER against CRE isolates producing Ambler class A β-lactamases, such as KPC- and KPC-3 has been also documented (Lob et al., 2017). Although many studies demonstrated an increased activity of antibiotics when used in combination against pathogens *in vitro*, little is known about the same efficacy *in vivo*, whereas some studies have shown that combined therapy could even be disadvantageous. The ESKAPE have been demonstrated to become resistant to either or both antibiotics used in combination due not only to natural selection of resistant strains but also to horizontal gene transfer from them to susceptible strains (Mulani et al., 2019). Therefore, further studies in establishing new potent inhibitor formulations and their validation in clinical trials are required, as well as extensive research of alternative strategies.

7.2.4 Emerging strategies as alternatives to antibiotics

In the last years new approaches to treating infections without directly killing the pathogens are also being investigated, including the inhibition of endotoxin production by the bacteria, deprivation of nutrients (e.g., iron), modulation of host response to microbes, and the use of probiotics to protect the host microbiota (Ventola, 2015). By using molecular genomics, genomes of organisms that produce natural antibiotics have been examined and biosynthetic pathways identified. Genetic manipulation of such pathways has produced new metabolites with antimicrobial activity and potential new

bacterial targets, including those that could reduce virulence (Piddock, 2012). Although several alternatives already exist in nature, the challenge is to implement them in clinical use. The next generation anti-infectives may be categorized in naturally occurring alternatives, synthetically designed strategies, and biotechnology-based strategies (Gosh et al., 2019), as summarized in Table 7.1.

Table 7.1 Classification of the novel alternatives to conventional antibiotics.

Naturally occurring alternatives	Synthetically designed strategies	Biotechnology-based strategies
Bacteriophages	Synthetic Mimics of Antimicrobial Peptides	Genetically Modified Bacteriophages
Antimicrobial peptides	Antibacterial Oligonucleotides	Lysins (Endolysins, Exolysins, and Autolysins)
Bacteriocins	Inhibitors of Bacterial Virulence	CRISPR-Cas 9
Probiotics		
Fecal transplant therapy		
Predatory bacteria		
Antibodies		

Phage therapy has been recognized as a promising alternative and selective approach to target resistant pathogens, offering numerous advantages such as high specificity, preventing damage to normal microbiota and eukaryotic cells, rapid proliferation in the bacterial host, low doses required for treatment (Domingo-Calap and Delgado-Martínez, 2018). However, phage therapy has some limitations. Bacteriophages' high specificity would require testing *in vitro* the efficacy against the disease-causing bacteria before administration, nevertheless the use of phage cocktails acting on a particular bacterial species has been designed to extend the spectrum of action (Chan et al., 2013). Another concern is related to the phages' safety, due to the possible release of endotoxins from the bacteria lysed by bacteriophages, as well as the carriage of virulence or resistance genes, and genetic elements involved in HGT within bacterial hosts (Casey et al., 2018). Furthermore, the pH, and thus stability, of the phage preparation needs to be considered in order to avoid the treatment's ineffectiveness. To overcome limitations of phage therapy, they can be combined with antibiotics which may show synergistic action by making either a phage or antibiotic or both to act more effectively (Mulani et al., 2019).

Antimicrobial peptides (AMPs) are produced by several multicellular organisms displaying antimicrobial, antibiofilm, antinflamatory, anticancer, antiplasmodial, insecticidal, spermicidal and immunomodulating activity (Gosh et al., 2019). The natural or bioengineered derivation makes them attractive candidates against MDR pathogens due to electrostatic interactions with cell membrane that lead to the inhibition of protein and nucleic acid synthesis, and final cellular lysis (Mahlapuu et al., 2016). AMPs are also known to modulate the immune system for defense against invading pathogens (Hancock et al., 2016). Basing on this evidence, peptides with no antibacterial activity but with antiendotoxin and immunomodulatory activities have been designed ("Innate defense regulatory peptides") and to date are at different stage of clinical trials (Mansour et al., 2015). Despite their successful *in vitro* and/or *in vivo* broad-spectrum activities, the *in vivo* efficacy of AMPs is challenged

by their cytotoxicity to mammalian cells, liability to degradation by tissue proteases, loss of activity at low salt concentrations or in presence of plasma proteins and higher production cost. Introduction of peptide mimetics or liposome encapsulation may improve their stability and reduce toxicity, while their combination with antibiotics or nanoparticles could enhance their efficiency (Mulani et al., 2019).

Nanomedicine is another emerging field with wide biomedical applications as antimicrobial agents. Silver nanoparticles (AgNPs) synthesized through physical, chemical or biological methods have shown promising antibacterial activity by releasing Ag+ ions which results in disruption of electron transport or signal transduction pathway or leading to generation of ROS, the latter involved in the damage of cell wall, cell membrane, cellular DNA, and/or proteins (Qayyum et al., 2017). However, data from *in vivo* studies aimed to test the toxicity, efficacy, pharmacokinetic, and immuno-modulatory response of the AgNPs are scant, and further research and clinical trials for their applications in wound dressings or medical devices are advocated.

Another promising strategy to combat resistant pathogens is represented by CRISPR/Cas (clustered, regularly interspaced, short palindromic repeats) system, which is a prokaryotic adaptive immune system against intruded heterologous DNA/RNA from virus or other organisms (Ding et al., 2020). In the key components of this bacterial immune system a 20 nt small RNA acts as a guide for Cas 9 (CRISPR-associated protein 9) to cleave foreign genetic elements, such as those present in plasmids and phages, at specific sites (Wang et al., 2016). Limiting the plasmid entrance into bacterial cells, this system may be exploited in order to reduce the transmission of AMR by HGT. So far, CRISPR technology has been used to increase the susceptibility of different Enterobacteriaceae by successfully decreasing the number of plasmids carrying the *bla*TEM-1 gene (Tagliaferri et al., 2020) and several studies have tried to program the CRISPR system to remove resistance genes or plasmids by genomic editing in *P. aeruginosa*, showing that CRISPR-Cas-based systems offer an excellent alternative to antibiotics (Chen et al., 2018).

Although natural and synthetic alternative strategies are being investigated in human and veterinary medicine, little attention is paid to the antimicrobial effects of edible lipids, such as medium-chain fatty acids (MCFAs) and monoglycerides. Among MCFAs, lauric acid (LA) and its monoglyceride derivative, glycerol monolaurate (GML), exhibit the strongest antimicrobial activity. Coconut and palm kernel oils are considered the main sources of LA, however some edible insects (e.g., *Hermetia illucens*) are gaining interest as novel feed ingredients, due to the high amount of LA they contain, as well as their numerous bioactive components, which provide many additional benefits to animal health. Although the beneficial effect of both MCFAs and LA is gradually being recognized, their high content within insects and, consequently, their possible role as antimicrobials, has not been well-reported. The potential of insect lipids represents a topic of growing interest in the current global scenario where a sustainable and circular economy is strongly advocated and the AMR crisis represents one of the major challenges of this century.

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Chapter 8

Insect-derived Lauric Acid as promising alternative strategy to antibiotics in the Antimicrobial Resistance scenario*

*Based on the manuscript: Borrelli L, **Varriale L**, Dipineto L, Menna LF, Fioretti A, 2021. Insect Derived Lauric Acid as Promising Alternative Strategy to Antibiotics in the Antimicrobial Resistance Scenario. Front Microbiol 12, 620798.

8.1 Tackling the rise of antimicrobial resistance. Any lipidic alternatives?

A New York Times headline from 1945 reads "Penicillin's finder assays its future; Sir Alexander Fleming observed that improved dosage method is needed to extend use." Despite this early warning, today, antimicrobial resistance (AMR) represents a global-scale public threat, and the world is now on the cusp of a "post-antibiotic era." The research community is investing in various drug discovery strategies to develop new antimicrobial drugs, as conventional drug therapies are becoming increasingly ineffective and limited (Farha and Brown, 2019; Schultz et al., 2020). Every year, 700,000 patients die worldwide due to AMR, but the number could easily and dramatically reach 10 million by 2050 (Ghosh et al., 2019). Antibiotic abuse both in humans and animals has greatly contributed to an increase in AMR and has also caused the accumulation of these compounds in the environment by selecting resistant microorganisms and turning the environment into an enormous reservoir for AMR genes (Roca et al., 2015). Moreover, antibiotics misuse in animal production and the EU ban on their use in feed (Regulation EC/1831/2003) has led to an increase in the incidence of livestock disease and economic damage (Dabbou et al., 2020).

To date, numerous natural and synthetic alternative strategies are being investigated such as antibodies, bacteriophages, antimicrobial peptides, and alteration of the gut microbiota, predatory bacteria, or fecal transplant therapy (Kadouri et al., 2013; Aroniadis and Brandt, 2014; Mandal et al., 2014; Mahlapuu et al., 2016; Ghosh et al., 2019; Rello et al., 2019). Antimicrobial lipids, such as medium-chain fatty acids (MCFAs) and monoglycerides could also be a suitable alternative to antibiotics. MCFAs are originally an important component of the innate immune system in mammalian breast milk, skin, and mucosa and also induce host defense peptides expression in humans and animals (Zhou et al., 2019). Among MCFAs, lauric acid (LA) (Figure 1A) and its monoglyceride derivative, monolaurin (glycerol monolaurate, GML) (Figure 1B), exhibit the strongest antimicrobial activity. They can modulate intestinal health by regulating the level of IL-6 and TNF-a (Dabbou et al., 2020) and, not least, they are Generally Recognized As Safe (GRAS) by the United States Food and Drug Administration (Yoon et al., 2018). Their ability to destabilize the bacterial cell membrane makes them promising candidates among novel antimicrobials because these bacteria are also unlikely to acquire resistance to these compounds (Petschow et al., 1996; Schlievert and Peterson, 2012; Jackman et al., 2020).

Although the beneficial effect of MCFAs and LA is gradually being recognized, their content within insects has not been well-reported. Recently, insects have been receiving considerable attention as novel alternative feed ingredients because of their excellent nutritional properties and potential effects on animal health. They contain bioactive components, such as LA, antimicrobial peptides (defensins, cecropins, attacins, lebocins, lysozime proline-rich peptides, gloverins, and moricins) and the valuable biopolymer chitin, part of the exoskeletons of arthropods and chitosan, produced commercially by deacetylation of chitin which has antimicrobial, anti-tumor and immune-boosting properties (Sogari et al., 2019; da Silva Lucas et al., 2020; Moretta et al., 2020; Smets et al., 2020).

Among edible insects, *Hermetia illucens* is one of the main sources of LA (Spranghers et al., 2018). Thus, it may represent a good candidate given the growing market demand for edible insects as a new source of food, and also considering the need to find new strategies for antimicrobial resistance. Therefore, based on the studies presented so far, the perspectives for future applications of insect lipids might also be considered in human nutrition.

8.2 Antimicrobial effect of the Lauric acid and Monolaurin

A recent overview reports the emerging antimicrobial properties of fatty acids (FAs) and their relation to virulence and quorum sensing (QS), such as diffusible signal factors (DSFs), acyl-homoserine lactones, and autoinducer-2 systems. The suppression of the expression of QS-regulated genes, especially those related to virulence (e.g., synthesis of toxins, fimbriae, hyphae, etc.) and other non-QS targets (proteins involved in efflux pumps, oxidative stress, and ergosterol synthesis) make FAs a new paradigm to cope with drug-resistant bacteria (Kumar et al., 2020). Of these, medium-chain fatty acids (MCFAs) and their monoglycerides have a broad spectrum of microbicidal activity against a wide range of pathogens both *in vitro* and *in vivo*, including multidrug-resistant bacteria, enveloped viruses, algae, fungi, and protozoa (Bergsson et al., 2001; Hilmarsson et al., 2007; Yoon et al., 2018; Zhou et al., 2019; Heriyati et al., 2020; Welch et al., 2020).

In the 1970s, Kabara's group carried out a wide-ranging assessment of the antibacterial activities of FAs and contributed to define the modern-day field of antimicrobial lipids from a chemical viewpoint (Yoon et al., 2018). LA and GML (Fig. 8.1) represent the strongest antimicrobial agents in mammalian milk, they are also found in other natural sources such as coconut oil and are often used as nutritional supplements (Lieberman et al., 2006; Dayrit, 2015; Kim and Rhee, 2016).

Due to their amphipathic properties, MCFAs exhibit an antimicrobial activity through a membrane-lytic behavior causing increased cell permeability and cell lysis. In addition, MCFAs disrupt the electron transport chain either by binding to electron carriers or interfering with oxidative phosphorylation, which are vital processes for energy production in bacterial cells. Furthermore, MCFAs can directly inhibit membrane enzymes such as glucosyltransferase and also target other membrane-associated proteins (Yoon et al., 2018). In some in silico studies, LA has been proposed as a natural antibacterial agent via inhibiting the MurA enzyme, which is involved in bacterial cell wall biosynthesis (Heriyati et al., 2020). Galbraith and Miller (1973a,b) reported that the activity of LA was decreased by Mg^{2+} and Ca^{2+} ions and increased by lower pH, suggesting that the uptake of LA is modulated by physico-chemical properties of both the acid and the bacterial surface. MCFAs and monoglycerides mainly work in the micellar state. Monoglycerides form micelles at lower concentrations than MCFAs, which helps to clarify why monoglycerides are often more biologically potent than FAs (Jackman et al., 2020). Overall, the esterification of a fatty acid to its corresponding monoglyceride derivative enhances the antibacterial effect (Yoon et al., 2018).

Kumar et al. (2020) showed that LA inhibits the swarming motility of P. mirabilis in a dose-dependent manner and, at higher levels, acts on *Clostridium difficile* cell membranes and adhesins. LA can inhibit hemolysin expression, extracellular polysaccharides (EPS) and biofilm production through RsbA (a histidine-containing phosphotransmitter of two-component signaling system) dependent pathway.

Additionally, GML may almost completely kill the vegetative cells and spores of aerobic and anaerobic bacteria (Schlievert et al., 2018; Yang et al., 2018). GML also inhibits the production of staphylococcal toxic shock toxin-1 effectively and the expression of virulence factors including protein A, alpha-hemolysin, b-lactamase, and the induction of vancomycin resistance in *Enterococcus faecalis* by interfering with signal transduction (Projan et al., 1994; Ruzin and Novick, 2000).

MCFAs and monoglycerides have been suggested as natural compounds for the control of various foodborne pathogens (Kim and Rhee, 2016; Dhakal and Aldrich, 2020). Hovorková et al. (2018) discovered that the bactericidal effect of MCFAs did not exert inhibitory effects against gut commensal bacteria.

MCFAs and their monoglycerides have emerged as promising additives for replacing in-feed antibiotics and promoting sustainable animal-food production, enhancing growth performance and animal welfare (Jackman et al., 2020). Apart from the direct effects on intestinal microbiota, MCFAs could have positive effects on gut health, modulated by the degree of esterification. MCFAs can improve the intestinal morphology and function, through their beneficial effects on crypt cell renewal (Spranghers et al., 2018) and have also an immunomodulatory activity (Zhang et al., 2016). Indeed,

new evidence points out that incubation of lauric acid, also found in human sebum, enhanced the innate immune defense of human sebocytes by upregulating the gene and protein expression of β-defensin-2, one of the most represented antimicrobial peptides detected in the skin (Zhou et al., 2019). In addition, LA has even been indicated as a natural antibiotic against some dermal infections, such as acne, with no toxic effect on human sebocytes (Nakatsuji et al., 2009).
As natural molecules, fatty acids have great potential and their combination with antimicrobials could reduce multidrug-resistant bacteria (Kumar et al., 2020).

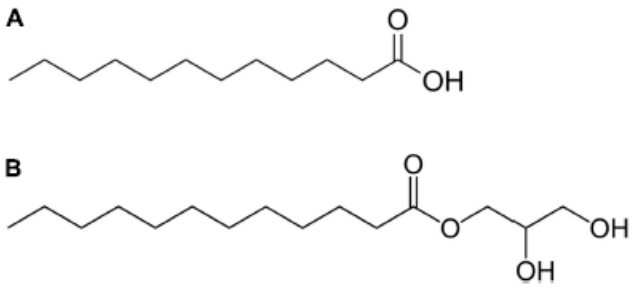

Fig. 8.1 Chemical structure of lauric acid (A) and glycerol monolaurate (B).

8.3 *Hermetia illucens* as one of the main sources of antimicrobial lipids

H. illucens (HI), a Diptera known as the black soldier fly (Sheppard et al., 2002) is a native of tropical, subtropical, and warm temperate zones of America. It is now widespread in tropical and warmer temperate regions between about 45°N and 40°S (Makkar et al., 2014). HI has been proposed since the 1990s as an efficient way to dispose of organic waste by converting it into a protein-rich and fat-rich biomass suitable for various purposes, including animal feeding, biodiesel, oil, and chitin production (van Huis et al., 2013).

For nutritional purposes, insects' fat was extracted from a limited number of species, i.e., HI, *Tenebrio molitor*, *Zophobas morio*, and *Bombyx mori*. Among these, HI has the highest amount of LA (up to 60%) (Spranghers et al., 2017), while in *T. molitor* it is less than 0.5% (van Huis et al., 2013; Gasco et al., 2019). HI oil also consists of various monoglycerides, diglycerides, and triglyceride, showing a very similar fatty acid profile and quality compared to that of coconut and palm kernel oil (Ushakova et al., 2016; Muller et al., 2017; Spranghers et al., 2017). LA concentration and synthesis may, however, undergo small variations according to the substrate used, to the shift from lipogenesis to glycogenesis related to the development stage, to the extraction methods, and the killing method and storage (Alifian et al., 2019; Caligiani et al., 2019; Rabani et al., 2019; Ewald et al., 2020; Kiero´nczyk et al., 2020).

The high fat content of the prepupae could limit their use as a feed ingredient. Thus, it could be interesting to partially extract the fat from the prepupal meal and use a sufficient amount of LA in the feed adding value to it, while the extracted part could be suitable for other purposes, such as the production of biofuel (Spranghers et al., 2017).

To reduce the costs for lipid extraction, the major challenges are large-scale production and legal frameworks to allow the use of insects as ingredients for food and feed. So far, insect lipids are allowed in feeding all animal species, but PAPs (processed animal proteins) are only permitted in aquaculture and the possibility of extending the authorization of their use to poultry and swine feed is still pending (Sogari et al., 2019).

HI is not considered a disease vector, since the adult fly is not attracted to human habitats or foods and the eggs are never laid on decaying organic material. Furthermore, the prepupae process organic waste very quickly and empty their digestive tract, limiting bacterial proliferation (van Huis et al., 2013; Makkar et al., 2014; Muller et al., 2017). Moreover, the larvae modify the microflora of manure, potentially reducing harmful bacteria such as *Escherichia coli* 0157:H7 and *Salmonella enterica* (Makkar et al., 2014).

HI is characterized by an immune system in which cell-mediated and humoral innate mechanisms work jointly. Hemolymph cells are involved in cellular immune responses, while phenoloxidase, antimicrobial peptides (AMPs), and proteins belong to humoral innate response (Zdybicka-Barabas et al., 2017). HI contains chitin which exhibits antimicrobial properties (Borrelli et al., 2017).

Up until recently, research has examined different strategies to take advantage of HI immunity. As reported, the modulation of the substrate where the larvae are fed could induce the expression of different proteins and specific immunity gene proteins with a different spectrum of antimicrobial activity (Zdybicka-Barabas et al., 2017; Vogel et al., 2018).

8.4 Antimicrobial properties of *Hermetia illucens* larvae inclusion in animal feeding

As natural and edible antimicrobial products, MCFAs have been proposed as alternatives to conventional antibiotic growth promoters in livestock nutrition (Fortuoso et al., 2019; Zhou et al., 2019). In the last few years, the use of insect-based diet as HIL meal and oil in substitution of the conventional feedstuffs has been investigated showing promising findings in terms of nutritive value and low environmental impact, with negligible effects on lipid digestibility, performance parameters, and animal health in the swine, poultry and rabbit industry (Cullere et al., 2016, 2018, 2019a; Dalle Zotte et al., 2018, 2019; Secci et al., 2018; Gariglio et al., 2019; Gasco et al., 2019; Yu et al., 2019, 2020).
To date, data on the antimicrobial effects of insect-based diet in vivo are scarce. All available studies are summarized in Table 8.1.

Table 8.1. Antimicrobial effect of insect-derived LA and GML in in vivo animal studies.

Elicitors	Animal species	Key findings	References
HI meal	Weaned piglets	2 log fold reduction of D-Streptococci	Spranghers et al., 2018
HI meal	Weanling piglets	Increased number of *Lactobacillus* and *Bifidobacterium*, quadratically decreasing number of *E. coli*	Yu et al., 2020
HI meal	Finishing pigs	Decreased abundance of Streptococcus spp., increased number of *Lactobacillus*, higher concentrations of total SCFA, upregulation of anti-inflammatory cytokine (IL-10)	Yu et al., 2019
HI oil	Growing rabbits	Significantly lower growth of *Yersinia enterocolitica*, positive influence on the cecal microbiota	Dabbou et al., 2020
HI meal	Siberian sturgeon	Positive effect on the gut microbiota composition and intestinal Morphology	Józefiak et al., 2019
HI oil	Young turkey	Reduced growth of Enterobacteriaceae, decreasing levels of IL-6	Sypniewski et al., 2020

8.5 Insect-based food perspectives in human nutrition

The nutritional values of available insects differ according to species, effects of diet or substrate, and environmental conditions. However, it is interesting that the value of some insect species in insect products is better than meat. The risk of consuming edible insects for humans compared with consuming other animal products or food protein sources is lesser-known. The EFSA has highlighted a lack of data regarding microbiology, virology, parasitology, and toxicology of edible insects. Currently, there is very limited information on the risks associated with families or species of insects, details of the manufacturing processes and the environmental impact of different farming systems, and there is also a lack of human consumption data (Ferri et al., 2019; van Huis, 2020).

Over recent decades, human nutrition has undergone dramatic changes such as an increased intake of partially hydrogenated oils and trans fatty acids, which may lead to a pro-inflammatory state, associated with obesity, type 2 diabetes, and other epidemic metabolic disorders in western countries (van den Brink et al., 2019).

The insect bioactive peptides and lipids have beneficial effects on human health, such as antioxidant, antimicrobial and antidiabetic properties, angiotensin I converting enzyme (ACE) inhibition activity, effects against inflammation and cancer. Insects can be also used as functional food ingredients (Dutta et al., 2019; da Silva Lucas et al., 2020).

Insect diet might also promote metabolic shifts, including the increased production of microbiota-derived short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate (Borrelli et al., 2017), exhibiting antibacterial activity against various pathogens (Yang et al., 2018). SCFAs improve mucosal and systemic innate and acquired immune responses to control inflammation during infections and reinforce homeostasis. Butyrate also inhibits histone deacetylase 3 to confer macrophages with non-inflammatory enhanced antimicrobial activity (Shinde et al., 2020).

As possible drawbacks for human nutrition, insect lipid fraction, unlike vegetable oils, is low in poly-unsaturated Fatty Acids (PUFA). As new alternatives to improve insect fats, the modulation of the substrate for the larvae by using fish-offal waste could increase the n-3 proportion in *Hermetia illucens* larvae meal and oil, as well as the direct inclusion of a PUFA rich feed ingredient (i.e., linseed) in animal diets, could be a useful strategy to provide healthier meat for human consumption (Cullere et al., 2019b; Caimi et al., 2020). Moreover, MCFAs show low solubility and unpleasant odors that stimulate the release of cholecystokinin and reduce the feed intake of animals. Encapsulation of MCFAs could be a suitable solution to overcome these limits (Zhou et al., 2019).

Due to their nutrient profile, insects could also be a game changer in the race to fight hunger, food insecurity, malnutrition (da Silva Lucas et al., 2020). There are many articles on the antimicrobial effects of GML *in vitro* and *in vivo* in animal studies but only three papers highlight in vivo antimicrobial effects in humans (Barker et al., 2019). However, other *in vivo* human studies and further research is recommended.

8.6 Concluding remarks

Antimicrobial resistance (AMR) is an impending public health crisis. As we respond to the COVID-19 pandemic, we are seeing what our health systems look like, with limited treatments available to tackle an outbreak. To stem the rise of AMR infections, physicians, veterinarians, and environmental scientists must all remain vigilant and maintain a one health view. This mini review shows how *Hermetia illucens* (HI) represent a good candidate source of LA with a potential role in the AMR scenario. In particular, HI-derived oil might be useful in protecting against microbial infections, modulating inflammation, healing wounds, and controlling the balance and distribution of bacteria in gut microbiota. The presence of insect-derived LA in animal feed could prevent the use of conventional antibiotics in meat production, limiting the diffusion of microbes harboring AMR genes (Dabbou et al., 2020).

If whole insects are used as feed, the antimicrobial molecules must remain stable during feed processing and digestion. Thus, these compounds could be purified and identified directly from larval extracts, or it would be possible to characterize *in vitro* HI immunity-related genes with the aim to discover novel antimicrobials and molecules (De Smet et al., 2018).

The MCFAs composition of HIL oil is very similar and can replace palm kernel or coconut oil, as additives and even pharmaceuticals (Matthäus et al., 2019; Rabani et al., 2019).

Both vegetable oils are products of plants from tropical climates, where they are abundant. The impact on the environment is also considerable, as an increase in demand for vegetable oils and biofuels contributes to water waste, tropical deforestation, loss in biodiversity, and habitat fragmentation. For this reason, palm kernel and coconut oil are currently criticized from an ecological point of view (Wang and Shelomi, 2017; Matthäus et al., 2019; Verheyen et al., 2020; Kiero´nczyk et al., 2020).

Therefore, in a vision of the circular economy, it would be possible to manage, reduce, and efficiently use organic waste for HIL rearing as a sustainable source of nutrients (according to the 4R of the EU Parliament Directive no. 2008/98 and the other Directive 94/62/EC).

The Hippocratic concept that “we are what we eat” is present in all cultures and it represents one of the biggest barriers to the consumption of insects in food neofobic western societies, which consider entomophagy to be disgusting (van Huis et al., 2013). The scientific community and all stakeholders must now make efforts to promote insects as a source of bioactive peptides and lipids and to prevent the next global threat of antimicrobial resistance as well as metabolic disorders.

If “we are what we eat,” but obesity, diabetes, cancer, and antimicrobial resistance are constantly increasing in western society, why not eat insects?

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Conclusions

Antimicrobial resistance is globally recognized as one of the major public health challenges of the twenty-first century, which will cost the lives of millions of people and health-care systems trillions of dollars if will not be addressed on a global scale. Although global actions have been undertaken, the problem still dominates because in many countries is not faced as a global priority due to limited economic resources, conflicts, political and health issues. This thesis outlined only a few aspects of the whole issue, underlining the need to consider the current challenges form a "One Health" perspective. Given the evidence that the human-animal-ecosystem interface represents a huge reservoir of antibiotic resistant bacteria (ARB), the development and implementation of national and international guidelines for the biological risk assessment of the emergence and dispersion of ARB in the environment is a strategic priority. If this goal is achieved and new drugs and novel therapeutic strategies are introduced, they could be effective over a longer period of time (Berendonk et al., 2015). Nevertheless, the application of new approaches to fight the AMR has so far been mainly at research level or at the preclinical setting, with limited number of clinical trials aimed at evaluating such strategies. The various drawbacks related to their development, as well as the poor return on investment, indicate that alternative approaches can only partially replace antibiotics and further research must be invested to fully assess and exploit their potential.

In closing, the current AMR crisis is likely to be a permanent feature of human society, with the COVID-19 pandemic posing additional threats to its management. Noteworthy, during the 1918 influenza pandemic the high mortality rates related to bacterial coinfections reflected the lack of available antibiotics, whereas in the current post-antibiotic and pandemic era the AMR crisis represents a major issue. Despite the Coronavirus disease had a great impact increasing the public awareness of AMR and the importance of prevention and infection control, on the other hand there are potential threats that could affect antimicrobial stewardship activities and drive antimicrobial resistance. First, hospital admissions increase the risk of health-care-associated infections and the transmission of multidrug-resistant organisms, which in turn lead to increased antimicrobial use. Second, disruptions to health services during the pandemic are causing interruptions to treatments, which can also lead to selection for drug resistance. Third, the high rates of inappropriate antimicrobial prescribing and the wide use of biocidal agents for environmental and personal disinfection, including in non-health-care settings, can lead to low level exposure to antimicrobial agents and consequently select for drug-resistant strains increasing the risk of cross resistance to antibiotics (Getahun et al., 2020). How the pandemic is directly impacting the overall levels of AMR has not yet been quantified, however efforts to prioritize antibiotic stewardship around the world must be redoubled. Managing this crisis and limiting its effects will be possible only through a shift in the global perception of antimicrobial use as well as in socio-ecological behaviors coordinated by implemented and sustainable global actions.

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