UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

ANTIBIOTIC RESISTANCE AND VIRULENCE PROFILES OF *ESCHERICHIA COLI* ISOLATED FROM CAPTIVE NON-DOMESTIC FELIDS

SOFIA ALEXANDRA QUELHAS CARAMUJO

ORIENTADORA:
Professora Doutora Maria Manuela Castilho Monteiro de Oliveira

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SOFIA ALEXANDRA QUELHAS CARAMUJO

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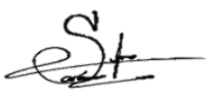

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Acknowledgements

Firstly, I would like to thank my supervisor, Professor Manuela Oliveira, for the continuous support and patience throughout this project and for giving me the freedom to base this dissertation on my love of big cats!

A special thank you to the team at the Big Cat Sanctuary, especially Emily, for welcoming me so warmly and for giving me the opportunity to develop this project as well as the chance to meet some of the most beautiful big cats! I am forever grateful for the experience!

To Fern and Will, thank you for your kindness and for allowing me to experience your incredibly gratifying but exhausting working days at Port Lympne!

To the amazing team at the Laboratory of Bacteriology, a big thank you for all your guidance and words of encouragement throughout my time at the lab. A very special mention to Raquel, without whom this project would not have been possible. Thank you for all your help, time and shared jokes and for making my time at the lab that much more enjoyable! My appreciation also goes out to all the students in our shared office for creating such a welcoming environment!

To Professor Gonçalo Pereira, for the indispensable contributions to the statistical analysis of this study.

To my FMV girls, my emotional support group; Inês, Bia, Maria and Catarina, thank you for all the memories throughout these years. For being with me throughout all the ups and downs and proving that this degree really isn't done alone. I couldn't have made it through this chapter of my life without you, and I hope we continue to be in each other's lives for many more years to come. To the boys who joined the emotional support group, better late than never, I am grateful for you and your friendship.

To my exam season girls, Teresa and Sara, thank you for the shared panic and mutual last-minute revision chaos, somehow, the collective despair made the nerves way more bearable!

To my uni godmother, Inês, for all the advice, guidance, and the friendship that developed along the way! Thank you for helping me navigate this degree!

To everyone else I had the pleasure of meeting throughout the past six years, thank you for the laughter, support and inspiration along the way.

And last but certainly not least, my biggest and most heartfelt thank you to my family. To my parents for supporting me always, even from far away, and allowing me to chase after my dreams. None of this would have been possible without your unwavering encouragement, and I am beyond grateful for you both. To my twin, always just a phone call away, offering a listening ear and a virtual shoulder to cry on, thank you for always being by my side.

PERFIS DE RESISTÊNCIA A ANTIBIÓTICOS E VIRULÊNCIA DE *ESCHERICHIA COLI* ISOLADA DE FELÍDEOS NÃO DOMÉSTICOS EM CATIVEIRO

Resumo

À medida que as iniciativas globais promovem a inclusão do conceito Uma Só Saúde na gestão integrada da saúde humana, animal e ambiental, compreender a resistência aos antimicrobianos (RAM) na vida selvagem continua a ser um passo essencial para a proteção da saúde pública e para a conservação da biodiversidade. Populações mantidas em cativeiro representam uma interface importante entre ambientes naturais e sob gestão humana, permitindo explorar como estes podem influenciar o perfil de resistência de bactérias sentinelas e potenciais riscos zoonóticos.

Este estudo pretendeu caracterizar os perfis de resistência e de virulência de isolados de *Escherichia coli* obtidos a partir de amostras fecais de felídeos selvagens mantidos em cativeiro. Foram incluídas neste estudo um total de 41 amostras fecais pertencentes a 11 espécies: leões (*Panthera leo*, n = 8), tigres (*Panthera tigris*, n = 5), jaguares (*Panthera onca*, n = 3), leopardos (*Panthera pardus*, n = 5), leopardos-das-neves (*Panthera uncia*, n = 4), chitas (*Acinonyx jubatus*, n = 3), pumas (*Puma concolor*, n = 3), servais (*Leptailurus serval*, n = 2), caracal (*Caracal caracal*, n = 1), gato-ferrugem (*Prionailurus rubiginosus*, n = 1), e leopardo-nebuloso (*Neofelis nebulosa*, n = 1). Após inoculação em meio selectivo, os isolados com morfologia compatível com *E. coli* foram identificados através do teste IMViC e caracterizados quanto ao seu perfil de suscetibilidade antimicrobiana relativamente a doze antibióticos, utilizando o método de difusão em disco. Os perfis de virulência dos isolados foram determinados fenotipicamente, através da deteção de produção de seis fatores de virulência: protease, DNase, gelatinase, lecitinase, hemolisinas e biofilme.

Foi possível isolar *Escherichia coli* a partir das 41 amostras em estudo (100%). Os isolados apresentaram níveis de resistência mais elevados relativamente à tetraciclina (19,4%) e à ampicilina (10,2%), sendo suscetíveis a metade (6/12) dos antibióticos testados. Um perfil de multirresistência foi observado em 9,3% dos isolados. A formação de biofilme (8,3%) foi o único fator de virulência detectado neste estudo. Associações estatisticamente significativas foram observadas entre o sexo do hospedeiro, a exposição antropogénica e as condições de alojamento no santuário, e o perfil de resistência a antibióticos específicos.

Os resultados sugerem que a manutenção em cativeiro influência os perfis de resistência antimicrobiana em felídeos selvagens, sublinhando a importância de integrar programas de vigilância da RAM e de adotar práticas de maneio que minimizem pressões seletivas e riscos de transmissão nestes contextos.

Palavras-chave: *Escherichia coli*; Felidae; cativeiro; resistência a antibióticos; factores de virulência

ANTIBIOTIC RESISTANCE AND VIRULENCE PROFILES OF *ESCHERICHIA COLI* ISOLATED FROM CAPTIVE NON-DOMESTIC FELIDS

Abstract

As global initiatives promote the inclusion of the One Health concept into the integrated management of human, animal, and environmental health, understanding antimicrobial resistance (AMR) in wildlife continues to be an essential step for both the protection of public health and the conservation of biodiversity. Captive wildlife populations represent a unique interface between natural and human-managed environments, contributing to the understanding of how captivity shapes indicator bacteria and potential zoonotic risk.

This study was conducted to characterise the profiles of antibiotic resistance and virulence of *Escherichia coli* isolates obtained from faecal samples of captive wild felids. In this study a total of 41 faecal samples were included belonging to 11 species: lions (*Panthera leo,* n = 8), tigers (*Panthera tigris*, n = 5), jaguars (*Panthera onca*, n = 3), leopards (*Panthera pardus*, n = 5), snow leopards (*Panthera uncia*, n = 4), cheetahs (*Acinonyx jubatus*, n = 3), pumas (*Puma concolor*, n = 3), servals (*Leptailurus serval*, n = 2), caracal (*Caracal caracal*, n = 1), rusty-spotted cat (*Prionailurus rubiginosus*, n = 1), and a clouded leopard (*Neofelis nebulosa*, n = 1). After inoculation in selective medium, isolates displaying morphology compatible with *E. coli* were identified through the IMViC test and characterised with respect to their antimicrobial susceptibility profile to twelve antibiotics, using the disk diffusion method. The isolates' virulence profiles were determined phenotypically and characterised by their ability to produce six virulence factors: protease, DNase, gelatinase, lecithinase, hemolysins and to form biofilm.

Escherichia coli was isolated from all 41 samples under study (100%). The isolates showed the highest resistance levels to tetracycline (19.4%) and ampicillin (10.2%), while complete susceptibility was observed in half (6/12) of the antibiotics tested. Multi-drug resistance was observed in 9.3% of the isolates. Biofilm formation (8.3%) was the only detectable virulence factor in this study. Statistically significant associations were observed between host sex, anthropogenic exposure and sanctuary housing conditions, and resistance profiles of specific antibiotics.

This study's findings suggest that captivity has an influence on the antimicrobial resistance profiles in wild felids, underscoring the importance of integrating AMR surveillance programs and adopting management practises which minimise selective pressures and transmission risks in these contexts.

Keywords: *Escherichia coli*; Felidae*;* captivity; antimicrobial resistance; virulence factors

PERFIS DE RESISTÊNCIA A ANTIBIÓTICOS E VIRULÊNCIA DE *ESCHERICHIA COLI* ISOLADA DE FELÍDEOS NÃO DOMÉSTICOS EM CATIVEIRO

Resumo alargado

A crescente prevalência de bactérias resistentes aos antimicrobianos (RAM) representa uma das maiores ameaças à saúde global, afetando não só a saúde humana e a animal, mas também a saúde ambiental. A abordagem Uma Só Saúde (One Health) reconhece a interligação intrínseca entre estes três setores e tem sido cada vez mais incorporada em iniciativas globais de conservação e saúde. Neste contexto, o estudo da RAM em populações de vida selvagem assume uma importância crítica, especialmente naquelas mantidas sob gestão humana.

Os centros de conservação de animais selvagens representam uma interface epidemiológica chave, atuando como potenciais pontos de encontro onde bactérias provenientes de humanos e do ambiente podem ser transmitidas à fauna selvagem. Embora a vida selvagem seja frequentemente considerada uma fonte de diversidade microbiana em ambientes naturais, as populações em cativeiro estão sujeitas a pressões antropogénicas únicas que podem moldar de uma forma significativa os seus perfis microbianos.

A vida de animais selvagens em cativeiro envolve a inevitável proximidade com humanos (médicos veterinários e tratadores) e, ocasionalmente, com animais de outras espécies mantidas no mesmo complexo, facilitando a potencial transmissão de estirpes bacterianas e de genes de resistência. Além disso, a gestão da saúde destes animais, muitas vezes com valor de conservação elevado ou com histórico de doenças prévias, exige intervenções veterinárias que recorrem à utilização de antibióticos. Este uso, embora essencial no tratamento de infeções, para a sobrevivência e para o bem-estar do indivíduo, constitui uma forte pressão seletiva para o desenvolvimento e manutenção de RAM na sua microbiota intestinal. Esta realidade diferencia as populações em cativeiro das suas congéneres selvagens, cujo contacto com antimicrobianos é tipicamente mais esporádico ou indireto.

Neste contexto, os felídeos não domésticos são de particular interesse. Dada a sua posição no topo da cadeia alimentar e os seus hábitos alimentares, são considerados potenciais reservatórios ou bioindicadores de contaminação ambiental, incluindo de RAM. A presença de bactérias resistentes nestas espécies em cativeiro não só compromete a eficácia dos tratamentos, o que constitui um desafio significativo para a medicina da conservação, como também representa um risco zoonótico ocupacional para os profissionais que com eles interagem. A eventual reintrodução destas espécies pode também favorecer a disseminação de estirpes resistentes para ecossistemas naturais, ou mesmo para outros locais, como por exemplo no âmbito de programas de reprodução *ex-situ*.

A implementação de *Antimicrobial Stewardship* (AMS) em contextos de medicina da conservação e em instituições de cativeiro apresenta dificuldades inerentes. A falta de dados robustos sobre a prevalência local de RAM, tal como dados farmacocinéticos e farmacodinâmicos em espécies selvagens obriga, muitas vezes, ao uso empírico de antibióticos de largo espetro, perpetuando o ciclo de resistência. Uma monitorização regular, como proposto neste estudo, torna-se assim vital para informar políticas de AMS ajustadas a estes ambientes particulares.

Neste cenário complexo, a espécie bacteriana *Escherichia coli* é universalmente reconhecida como um microrganismo sentinela crucial. Sendo um membro ubíquo da microbiota intestinal de mamíferos, *E. coli* é um excelente indicador da prevalência de RAM, refletindo a exposição ambiental e as pressões seletivas no trato gastrointestinal. A caracterização da RAM e da expressão de fatores de virulência em isolados de *E. coli* provenientes de felídeos em cativeiro fornece informações importantes para melhor compreender as dinâmicas de resistência e apoiar estratégias preventivas que minimizem o risco de transmissão e o impacto da RAM na medicina veterinária e na saúde pública.

Apesar da investigação existente sobre a resistência antimicrobiana em animais selvagens, subsiste uma compreensão limitada sobre a forma como as condições em cativeiro influenciam os perfis microbianos dos animais sob cuidado humano, e como tais alterações podem acarretar implicações para a saúde animal, os esforços de conservação e potenciais riscos zoonóticos.

Este trabalho teve como objetivo principal investigar a prevalência e os padrões de resistência antimicrobiana e dos fatores de virulência fenotípicos em isolados de *E. coli* obtidos a partir de espécies de felídeos não domésticos mantidos em cativeiro. Como objetivo secundário, este estudo procurou comparar os perfis de resistência e virulência entre indivíduos e espécies distintas da família Felidae, com o intuito de avaliar o papel que o cativeiro desempenha na modelação da resistência bacteriana e da patogenicidade. Para tal, variáveis independentes relacionadas com o cativeiro e características individuais do animal foram estatisticamente analisadas quanto à sua potencial influência nos perfis de resistência. Este trabalho visa contribuir com uma perspetiva adicional para a informação disponível sobre RAM, fornecendo informações específicas relacionadas com as espécies de felídeos e abordando o cativeiro como um fator determinante de resistência antimicrobiana.

Para cumprir os objetivos definidos foi delineado um estudo, realizado ao longo de quatro meses, que incidiu sobre a avaliação da presença de *E. coli* em felídeos selvagens em cativeiro. Foram analisadas um total de 41 amostras fecais, obtidas a partir de 11 espécies de felídeos não domésticos alojados num santuário de felídeos selvagens. As amostras foram recolhidas de forma oportunista e não invasiva a partir de fezes depositadas nos respetivos alojamentos, e a partir da porção interior do material fecal para minimizar a contaminação

ambiental. As amostras fecais foram inoculadas em meio de cultura seletivo, e os isolados com morfologia compatível com *E. coli* foram submetidos a testes bioquímicos, sendo a sua identificação confirmada através da realização do teste IMViC, um método padrão para a diferenciação de bactérias da família *Enterobacteriaceae*. Foi obtido um total de 108 isolados de *E. coli* para subsequente análise.

A suscetibilidade dos isolados de *E. coli* a um painel de doze antibióticos representativos de diferentes classes foi avaliada utilizando o método de difusão em disco (Kirby-Bauer), de acordo com as diretrizes do "Clinical and Laboratory Standards Institute" (CLSI), permitindo a classificação dos isolados como suscetível (S), intermédio (I) ou resistente (R) aos antimicrobianos testados. Os isolados com um perfil de não-suscetibilidade adquirida a pelo menos um agente em três ou mais classes de antimicrobianos foram classificados como Multirresistentes (MDR).

Os perfis de virulência de cada isolado foram determinados fenotipicamente, utilizando meios diferenciais para a deteção de produção de seis fatores de virulência relevantes para a patogenicidade de *E. coli*: protease, DNase, gelatinase, lecitinase, hemolisinas e biofilme.

Para além da determinação da prevalência de resistência e virulência, a análise estatística dos dados permitiu explorar as potenciais associações entre as características dos isolados e as variáveis independentes relacionadas com o indivíduo e as condições em cativeiro. As variáveis independentes testadas incluíram a espécie, o grupo taxonómico (Pantherinae vs. Felinae), a origem, o sexo, a idade, o tempo de permanência no santuário, o tipo de alojamento (individual ou partilhado), o tratamento antibiótico nos últimos seis meses, o estatuto de conservação segundo a IUCN e o fator de proximidade humana (incluindo quatro variáveis detalhadas na Tabela 1).

Foi possível isolar *E. coli* a partir de 100% das amostras em estudo (n=41), o que reitera a universalidade desta bactéria como comensal no trato gastrointestinal de mamíferos.

Relativamente à suscetibilidade antimicrobiana, os isolados demonstraram ser suscetíveis a metade dos antibióticos testados (6/12). Contudo, foi observada uma resistência moderada a agentes antimicrobianos historicamente muito utilizados em medicina veterinária e humana, como a tetraciclina (19,4%) e a ampicilina (10,2%). A resistência à tetraciclina é frequentemente observada em estudos de RAM em animais selvagens, refletindo o seu uso prolongado e generalizado, e a fácil disseminação dos genes de resistência associados. A resistência à ampicilina também é um indicador comum de pressão seletiva.

Um perfil de MDR foi observado em 9,3% dos isolados. Embora esta percentagem seja moderada, indica que os felídeos em cativeiro não estão isentos do problema de MDR, e que a pressão seletiva no ambiente de cativeiro é suficiente para selecionar e/ou manter estas estirpes.

A avaliação fenotípica dos fatores de virulência revelou um perfil de baixa patogenicidade nos isolados de *E. coli* avaliados, sendo que a capacidade de formação de biofilme (8,3%) foi o único fator de virulência detetado neste estudo.

Foram observadas associações estatisticamente significativas entre características específicas do hospedeiro e as condições de cativeiro com o perfil de resistência a antibióticos específicos. Nomeadamente, o sexo do hospedeiro, a exposição antropogénica e as condições de alojamento no santuário, foram identificados como tendo um potencial papel modulador na emergência e seleção de determinados perfis de RAM.

Em suma, os resultados sugerem que a manutenção em cativeiro influencia os perfis de resistência antimicrobiana em felídeos selvagens, expondo-os a pressões seletivas que resultam na emergência e potencial disseminação de isolados resistentes, incluindo estirpes multirresistentes. Estes dados sublinham a importância de integrar programas de vigilância da RAM nestes contextos, e de adotar práticas de maneio que minimizem as pressões seletivas e os riscos de transmissão.

É ainda importante referir que a interpretação dos perfis de resistência e dos fatores de virulência observados neste estudo deve ser cautelosa, dadas as limitações inerentes ao desenho experimental e à natureza da amostragem. Além disso, as diferenças nas práticas de maneio aplicadas no santuário, no *design* dos alojamentos e nas rotinas de gestão restringem a extrapolação destes resultados e a reprodutibilidade para além desta instalação.

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AMR Antimicrobial Resistance or Antimicrobial-resistant

AMS Antimicrobial Stewardship

AMU Antimicrobial Use

ARB Antimicrobial Resistant Bacteria

ARG Antimicrobial Resistant Genes

BIAZA British and Irish Association of Zoos and Aquariums

BHI Brain Heart Infusion

CLSI Clinical and Laboratory Standards Institute

CPSG Conservation Planning Specialist Group

EAZA European Association of Zoos and Aquaria

ESBL Extended-Spectrum Beta-Lactamase

EU European Union

HGT Horizontal Gene Transfer

IMViC Indole, Methyl Red, Voges-Proskauer, Citrate

IUCN International Union for Conservation of Nature

MAR Multiple Antimicrobial Resistance

MDR Multi-drug Resistance or Multi-drug Resistant

MRVP Methyl Red Voges-Proskauer

PD Pharmacodynamics

PK Pharmacokinetics

QAC Quaternary Ammonium Compounds

SIM Sulphide Indole Motility

STEC Shiga toxin-producing Escherichia coli

US United States

V. Index Virulence Index

WAZA World Association of Zoos and Aquariums

WHO World Health Organization

WOAH World Organization for Animal Health

ZIMS Zoological Information Management System

Section 1. Internship Report

1.1 Big Cat Sanctuary and Port Lympne Safari Park

My internship began at the Big Cat Sanctuary, located in the southeast of England, where I was granted the opportunity of visiting the facilities and collecting the biological samples and contextual data needed for my research project. Over the course of four days on site, adding up to approximately 35 work hours, I was able to gain insight into the sanctuary's operations, including the daily management practises involved in the care of captive non-domestic felids and in the educational activities available to the public.

During my short time at the sanctuary, I observed and participated in the standard workday, which typically spanned approximately 8.5 hours. The tasks to be performed were distributed by the animal care staff. In the absence of an in-house veterinarian, I shadowed the animal keepers as they carried out daily husbandry activities encompassing feeding preparation, enclosure maintenance and health checks.

My hands-on involvement included preparing and weighing individualised meat-based diets, assisting with the feeding routines, as well as the maintenance and cleaning of the animal enclosures. I also contributed to the development and implementation of cognitive and sensory enrichment as part of husbandry practises. Additionally, I observed the preparation and administration of oral medications and tailored dietary supplements as part of the specific needs of different species. As part of the sanctuary's engagement with the public, I was also able to shadow guest experiences facilitated by the animal care staff.

During this time, the collection of samples for my project was integrated into the animals' daily husbandry routines to minimize disruption, with the last hours of the afternoons dedicated to data extraction and review using the Zoological Information Management System (ZIMS) database.

In addition to my time at the Big Cat Sanctuary, I also had the opportunity to visit Port Lympne Safari Park located nearby. Over the course of two days, I shadowed zookeepers from two animal care departments: the Primate Section and the Ungulate/Safari Herbivore Team. My involvement included assisting with the routine husbandry tasks such as meal preparation and enclosure maintenance and cleaning. On the first day, I was also able to visit the on-site Veterinary Department, where I assisted with the anaesthesia monitoring of a squirrel monkey undergoing a vasectomy procedure.

These experiences broadened my understanding of species-specific care, and the multidisciplinary collaboration required for zoological health management.

1.2 Laboratory of Microbiology and Immunology of the Faculty of Veterinary Medicine, Lisbon, Portugal

The second half of my internship took place at the Laboratory of Microbiology and Immunology of the Faculty of Veterinary Medicine, which spanned approximately four months adding up to a total of approximately 600 work hours, from late September 2024 to the end of January 2025. With the valuable guidance and support from the microbiology team throughout this period, this phase was primarily dedicated to the laboratory analysis of the faecal samples collected from the non-domestic felid collection at the Big Cat Sanctuary.

In the early stages of the internship, I outlined a tailored laboratory protocol to guide the microbiological testing of the samples. I worked independently to manage my own schedule and daily tasks which typically required an average of 7 hours per day to complete. My responsibilities included the preparation and distribution of various culture media, inoculation of samples, bacterial propagation techniques, and macroscopic and microscopic assessment of bacterial cultures. I also conducted a series of biochemical tests to support the identification of the isolates.

Subsequently, standardized disk diffusion methods and phenotypic plaque assays were performed to determine antimicrobial susceptivity profiles and virulence phenotyping of the bacterial isolates. A comprehensive overview of these methodologies can be found in Section 3: Materials and Methods.

Parallel to the laboratory work, I continuously organized data to facilitate subsequent statistical analysis and interpretation of results.

Section 2. Literature Review

2.1 The Importance of Wildlife Conservation Centres and Zoos

With the first zoos having been founded in a different world than the one in which we live in today, the value of such conservation centres has often been underestimated and frequently criticized by the public (Spooner et al. 2023). This is why, as we find ourselves in the midst of a climate and biodiversity crisis (Bradshaw et al. 2021), we need to acknowledge the potential of these organizations and work on the challenges they face that are impacting the role they play on biodiversity conservation and the future of this planet.

Nowadays, more zoos and conservation centres are increasingly aspiring to meet wider conservation requirements established in strategies such as the One Plan Approach, first introduced by the Conservation Planning Specialist Group (CPSG) in 2011. This long-term action plan, endorsed by The World Association for Zoos and Aquariums (WAZA), calls for an integrated species conservation strategy. It emphasises collaborative conservation efforts to be applied to all species populations, both *in-situ* and *ex-situ*, under a single comprehensive management plan striving towards a common goal, namely, to establish viable populations living in sustainable ecosystems (Conservation Planning Specialist Group 2025).

At the heart of wildlife conservation lies the preservation of endangered species facing a multitude of threats including habitat destruction, human-wildlife conflicts, private wildlife ownership and illegal wildlife trading (International Fund for Animal Welfare 2024). To manage some of these threats it is necessary to find a way for humans and animals to coexist without having to make sacrifices that compromise them. Behind a stable and sustainable ecosystem lies a myriad of species, each playing a unique part, rendering wildlife protection as an essential step towards ensuring the health and survival of our planet (Sillero-Zubiri et al. 2023).

Community engagement and education initiatives are two critical aspects involved in the role that conservation centres and zoos actively play in biodiversity conservation. Even with the increase in accessibility and global connectivity in today's word, for most people wildlife sanctuaries remain the only real-life exposure to wildlife. These experiences provide the fundamental foundations for the development of an interspecies connection, both emotionally and intellectually, necessary to guarantee support of the conservation efforts implemented by wildlife organisations (Greenwell et al. 2023).

As a part of the British and Irish Association of Zoos and Aquariums (BIAZA), member associations, like the Big Cat Sanctuary, strive to have a significant contribution to the protection of biodiversity, with research being an integral part of the activities of this association. In fact, BIAZA maintains a research database on the projects undertaken by their

members focusing on their *ex-situ* animal collections (British and Irish Association of Zoos and Aquariums 2025). In this manner, recent studies have highlighted how the growth rate of research activity by BIAZA associations has visibly increased in the past 20 years (Hosey et al. 2019), leading to an increase in publication outputs, scientific knowledge and organization credibility (Loh et al. 2018). The relevance of the scientific contributions of zoos to the support of species conservation is also firmly embedded in other associations' research strategies, such as the European Association of Zoos and Aquaria (EAZA) and the World Association of Zoos and Aquariums (WAZA), showcasing the importance of zoo-generated data and the vast amounts of relevant research involving these organisations (Kögler et al. 2020). This evidence highlights that animals in *ex-situ* collections should be classified as being of high scientific value, besides being important ambassadors for education initiatives and key in raising awareness to conservation issues impacting their wild counterparts (Poo et al. 2022).

Additionally, a central aspect of zoo-based research is the opportunity for research training (Hosey et al. 2019). The activities performed within these institutions allow students to practise and develop key skills and be involved in research projects. If properly supervised, student projects can influence the management of species (Rose 2014) and reinforce the need for collaborations between academia and zoological collections (Rose et al. 2019).

In addition to engaging in research projects, these institutions also contribute to *in-situ* conservation initiatives while also participating in *ex-situ* conservation projects. These include captive breeding of endangered species and animal relocations between similar organisations in the context of genetic diversity (Fatima 2024). The report of success stories involving reintroduction projects worldwide, including the reintroduction of the Grey Wolf to Yellow Stone National Park in 1995 (Boyce 2018), of the Iberian Lynx in the Iberian Peninsula (IUCN 2024) and of the African Wild Dog in South Africa (Bouley et al. 2021), demonstrate the importance of conservation efforts in restoring species populations and contributing to ecological balance and biodiversity. The most recent overview of EAZA-managed *ex-situ* programs includes a broad range of threatened taxa; among these, several big cat species such as the Amur leopard, a known flagship species, the Sumatran tiger, the African cheetah and the Snow leopard (EAZA 2025), around which many of these programs are centred, showing the importance of sustained commitment to projects involving the Felidae family.

On the other hand, in a rapidly changing society, it has become increasingly more challenging to obtain public support, which remains vital for both the reputation and effective operation of conservation organizations (Miranda et al. 2025). Nevertheless, the donations and support received from the public through global campaigns, such as the 'Big Cats in Crisis' campaign by the Big Cat Sanctuary (The Big Cat Sanctuary 2024), the 'Save Our Zoo' campaign by Chester Zoo (Ferrer and Katanich 2020), and various EAZA conservation campaigns (EAZA 2025), clearly highlight the value that zoos bring to society. Nonetheless,

while these campaigns showcase the positive impact and importance of public engagement, they also indicate the vulnerability of these organizations and how deeply reliant they are on continued public support (Spooner et al. 2023).

Although zoological institutions and conservation centres are increasingly recognized for their roles in education, scientific research, and the preservation of endangered species, it is important to acknowledge that not all zoos and wildlife centres consistently meet the same standards of animal welfare and conservation practices. Variability in resources, management, and regulatory aspects mean that the quality and impact of these institutions can significantly differ (Kim, Choi, et al. 2024). Moreover, quantifying the precise conservation contributions of zoos remains a complex and challenging task, given the multifaceted nature of their roles (Loh et al. 2018). Addressing these challenges through the reinforcement of welfare and conservation standards, alongside transparent reporting and independent evaluation, is essential. Such measures, as the newly published standards of modern zoo practice covering Great Britain (DEFRA 2025), will help to secure continual public and scientific support, ensuring that zoos and conservation centres can effectively contribute to global biodiversity preservation and the restoration of natural ecosystems.

2.2 One Health Implications of Captive Wildlife Management

2.2.1 Public Health Concerns in Wildlife Conservation Centres and Zoos

Although public health is receiving increasing attention within veterinary medicine in the context of zoos and wildlife conservation centres, this multifaceted field is still occasionally regarded as a ‘relatively new focus for zoos’ within which these organisations are perceived as having limited influence (Spooner et al. 2023). Conversely, the World Organisation for Animal Health (WOAH) and the International Union for Conservation of Nature (IUCN) (2024) recognize how wildlife in captive settings, such as zoological collections and wild animal sanctuaries, can be a valuable source of information on wildlife diseases and pathogens both for surveillance and monitoring purposes.

The majority of scientific literature published with a focus on public health within wildlife sanctuaries and zoos describe zoonotic risks as their primary concern (Kvapil et al. 2021; Thal and Mettenleiter 2023; Goulet et al. 2024; Kuhn et al. 2024; Rampacci et al. 2024). Wildlife populations are classified as reservoirs for zoonotic pathogens and around 70% of zoonotic emerging infectious diseases stem from wildlife (Robinette et al. 2017; Hirst and Halsey 2023; Kuhn et al. 2024). With regard to wildlife-housing institutions, this risk has been shown to be

influenced by a rise in human activity (Esposito et al. 2023; Van Leeuwen et al. 2023), which, in turn, increases the risk of pathogen spillover. On the other hand, the rise in pathogen detection may also be explained by the increased application of One Health measures and stricter surveillance policies within zoological organisations (Van Leeuwen et al. 2023). Minimizing interactions between humans and wildlife may be one of the most effective strategies to reduce pathogen transmission, including the spillover of novel pathogens between species. However, complete separation is not always possible. In situations where these interactions cannot be reduced or avoided, targeted management interventions can help mitigate the risks, reducing the likelihood of interspecies disease transmission (Hopkins et al. 2024).

In a study by Goulet et al. (2024), the idea that wildlife health requires a targeted management approach is explored due to the various ways in which humans use and interact with animal populations. The authors discussed the need for an integrated strategy regarding the implementation of the One Health concept and stressed the importance of an 'operational framework' to facilitate the proposal of clear guidelines by policymakers and to ensure an effective path towards the management of wildlife health.

As part of broader policymaking efforts, particularly those led by the organizations from the Quadripartite Alliance, to address emerging health challenges at the human–animal–environment interface, the One Health Joint Plan of Action (2022–2026) was established to complement existing One Health initiatives, such as those highlighted at the IUCN World Conservation Congress in 2020 (IUCN 2022). This plan outlines a comprehensive framework focusing on enhancing disease prevention, surveillance and response, being highly relevant to zoos and wildlife sanctuaries (World Health Organization et al. 2022). These institutions are recognised as housing sites for a diversity of wildlife species, providing a crucial and unique opportunity for the early detection of pathogens aiming to reduce the risk of zoonotic spillover and contribute to the monitoring of emerging or re-emerging zoonotic diseases (Van Leeuwen et al. 2023). Through these institutions, key surveillance systems and response protocols to emerging diseases can be introduced, and prudent antimicrobial use policies implemented within animal care. This plan of action contributes to the safeguarding of animal welfare and also plays a pivotal role in public health.

Several zoonotic bacterial pathogens are considered a major global health concern, including antimicrobial resistant strains. Antimicrobial resistance has been consistently classified by the World Health Organisation (WHO) as a major health problem at international level (World Health Organization 2023), with recent reports highlighting the ongoing challenges in combating antimicrobial resistance (AMR) worldwide (World Health Organization 2025). The WHO's Bacterial Priority Pathogen List (2024) recently classified the bacterial pathogens considered of highest importance to public health and crucial for the development of strategies

to control the dissemination of resistant strains, namely carbapenem-resistant and third generation cephalosporin-resistant Enterobacterales and carbapenem-resistant *Acinetobacter baumannii.* On the other hand, the Compendium of Measures to Prevent Disease Associated with Animals in Public Settings (Daly et al. 2023) identifies the most common pathogens from animals housed in public settings which pose a risk to human health. In this document, wild animals are recognised as sources of enteric disease outbreaks, including *Escherichia coli* infections, cryptosporidiosis, campylobacteriosis and infections by non-typhoidal *Salmonella enterica.* Furthermore, zoological institutions are further highlighted as public settings where opportunities for zoonotic disease exposure may occur.

Zoo-housed captive wildlife has been recognised as a source of transmission for various zoonotic bacterial pathogens which could lead to infections in animal keepers, zoo workers and potentially visitors (Esposito et al. 2023; Schmartz et al. 2024). The most common transmission routes of pathogens involving animals in captivity include faecal-oral transmission, direct contact and indirect contact through insect vectors and contaminated objects. This can occur when people touch, hold, feed or are licked by these animals (NASPHV et al. 2023; Rampacci et al. 2024). Present scientific literature provides species-diverse documentation of zoonotic bacterial transmission and infectious diseases acquired from captive animals. The focus of this review will be on the order Carnivora, more specifically the Felidae family.

Wild felids are considered major reservoirs for a large variety of zoonotic bacterial pathogens such as *Mycobacterium bovis, Salmonella* spp., *Escherichia coli* and *Leptospira* spp. (Straub et al. 2021; Gumbo et al. 2023; Li, Lan et al. 2024; Rampacci et al. 2024). A literature review focusing on wildlife hosts of specific zoonotic bacterial pathogens described the presence of 26 pathogenic species in the lion (*Panthera leo*), making it one of the species within the Carnivora order with the greatest pathogen richness (Hirst and Halsey 2023). A study by Iatta et al. (2020) on infectious agents of zoonotic concern in captive tigers described bacterial infections by *Rickettsia conorii, Bartonella henselae* and *Leptospira interrogans* in these animals. Similarly, Žele-Vengušt et al. (2021) analysed the exposure of free-ranging wildlife from Slovenia to *L. interrogans* and found that large carnivores had the largest proportion of positive samples. Additionally, a study on non-typhoidal *Salmonella enterica* isolated from captive large felids detected *Salmonella enterica* subsp. *enterica* as the most prevalent subspecies (42.7%) found predominantly in felids housed in circuses. In this study, animals from zoos and rescue centres had a lower number of serovariants which, in turn, demonstrated a lower proportion of resistant isolates to the antimicrobials tested in comparison, potentially due to the increased contact with humans in circus settings (Rampacci et al. 2024).

The transmission of infectious diseases of diverse etiological origin from wildlife to humans has also been documented in the past (Chomel et al. 2007; Mobo et al. 2010; Grob et al. 2018; Green et al. 2020), together with cases of anthroponosis from zookeepers to animals (McAloose et al. 2020; Sangkachai et al. 2022). These findings highlight the need for continued research into the dynamics of disease transmission within a zoological setting and reinforce the importance of zoos as critical platforms for the study of zoonotic risks and advancing evidence-based practices that support public health at the human–animal interface.

These zoonotic and anthroponotic interactions further underline the necessity of applying a One Health framework to zoological environments, where among the most pressing threats in this context is the rise of AMR. Addressing AMR in *ex-situ* wildlife settings requires not only surveillance but also coordinated mitigation strategies. Thal and Mettenleiter (2023) outlined several interventional measures to reduce the risks of AMR, including the optimization of antimicrobial use, ensuring an adequate supply of antibiotics and access to diagnostic capacities. However, while these measures may be effective theoretically, their successful implementation may be limited in under-resourced sectors such as animal sanctuaries, rehabilitation centres and zoos which, as mentioned previously, are heavily reliant on public support. In these cases, the publication of new regulations without accompanying support systems is insufficient.

2.2.2 The Influence of Captivity on the Development of Antimicrobial Resistance in Wildlife

While strongly influenced by antibiotic use, AMR has been previously well documented as a naturally occurring phenomenon (Perry et al. 2016). However, human-related factors, such as those associated with wildlife living in captivity, may further contribute to the prevalence of antimicrobial resistance in the form of antibiotic-resistant bacteria (ARB) as well as antibiotic-resistant genes (ARG) (Huang 2022).

2.2.2.1 Anthropogenic Pressures

Close human-animal interactions may cause the transmission of AMR directly from humans to wildlife and vice versa or indirectly via co-used environments. This knowledge led to the development of studies dedicated to investigating the impact of human presence on AMR in wild animals living in captivity (Tanga et al. 2024).

Existing literature, specifically on non-human primates, suggests the potential for anthropogenic transmission of resistant bacteria to captive great apes, our closest living

relatives (Schaumburg et al. 2012; Bager et al. 2022). This evidence supports the hypothesis that captivity may contribute to higher levels of antimicrobial resistance compared to those observed in free-ranging wild animals living in environments with minimal human presence. Baros Jorquera et al. (2021) investigated anthropogenic influence in animal rehabilitation centres, aiming to understand the influence of human exposure to wildlife populations in these settings. settings. In this study, a positive correlation was found between prolonged captivity and a higher proportion of resistant Enterobacteriaceae isolates, which may have been caused by an increased frequency of proximity to humans as well as a longer antibiotic treatment plan. However, no association was found between antibiotic treatment and frequency of resistant isolates. Furthermore, this study cited an evaluation of elephant seals in captivity, which found a higher proportion of animals with antibiotic resistant *Escherichia coli* at the time of release from rehabilitation in comparison with at admission time (Stoddard et al. 2009), corroborating the positive correlation previously mentioned.

Interestingly, wild animal groups under increased anthropogenic pressure in Africa can be distinguished by the comparison of gut microbiomes, highlighting the impact of human-animal interactions even in free-living wildlife populations (Gomez et al. 2015). Complementary evidence from China indicates that human disturbance can also shape the resistome, with wildlife previously exposed to human activity harbouring ARGs at a higher relative abundance and greater diversity index compared to wildlife living in 'wild habitat environments' (Zou et al. 2023).

In captivity, animals are often housed in environments that are cleaned, managed, and shared by humans, leading to an increased likelihood of microbial exchange (Huang 2022). As such, in zoos and wildlife rehabilitation centres, where frequent human interaction is unavoidable, captive wildlife has been shown to harbour human-associated microbiota acquired through indirect transmission, frequently including ARGs (Tsukayama et al. 2018). Curiously, some studies have compared the microbiomes of captive animals and humans and found a closer resemblance between them than to those of the same free-roaming animal species. This has led to research exploring the term 'humanization' of the gut microbiota and of the resistome of certain animal species, suggesting that anthropogenic influence in captivity fundamentally shapes the microbial communities of these animals (Clayton et al. 2016; Tsukayama et al. 2018; Wang et al. 2021). For example, in a study on black capuchin monkeys, ARGs commonly observed in clinical and environmental enterococci isolates were found at higher frequencies in samples from animals in captivity compared to the wild monkeys. This suggests that captivity may facilitate the acquisition of resistance-related genes, allowing captive animals to unintentionally act as amplifiers of antimicrobial resistance resulting in the continued dissemination of these genes in the environment, possibly contributing to the persistence of resistance over time (Grassotti et al. 2018). In fact, related studies have

described the presence of genetically identical resistant *E. coli* strains across multiple species within a single zoo, which is suggestive of clonal expansion (De Witte et al. 2021). This may indicate that shared sources such as food or enclosure surfaces may act as common reservoirs for AMR transmission. Indirect acquisition of resistance through environmental exposure was also identified as a potential explanation for the high prevalence of resistant bacteria in free-living wildlife inhabiting anthropogenically contaminated areas by previous authors (Brisson et al. 2023).

Additionally, restricted access to diverse habitats and strict restrictions on interspecies interactions may result in captive animals being exposed to a narrower range of environmental microbes. This limited exposure is reflected in a lower abundance and richness of bacterial taxa in these animals (McKenzie et al. 2017) and may impair resistance to colonization, creating the opportunity for potential resistant bacteria to establish and persist. Supporting this, some researchers have identified captivity itself as the main factor influencing the diversity and abundance of ARGs within the host's gut microbiota (Huang 2022). This concept has been previously referred to as the 'built environment effect', highlighting how human-made habitats, such as animal enclosures in zoos or wildlife rescue centres, can shape the microbial communities of resident animals (Trinh et al. 2018).

2.2.2.2. Antibiotic Use

Given that antibiotic use is the primary driving factor in the emergence and spread of AMR worldwide (Estany-Gestal et al. 2024), it has been proposed that the development of resistant bacteria is likely proportional to the intensity of human activities, particularly those involving the conservation and welfare management of species in the context of captivity (Brisson et al. 2023). In these settings, antibiotic use is difficult to monitor given the absence of species-specific antimicrobial protocols and the lack of guidelines tailored to wildlife. Consequently, the need for such policies is widely recognised in this field of veterinary medicine with existing studies highlighting the benefits of having standardized antimicrobial practises in place (Miller et al. 2024).

Wildlife receiving medical care can be found in a wide range of settings, where antimicrobial use practises must be adjusted to suit the organizations' conditions. Due to limitations in funding and resource availability, as well as the experience and preference of veterinary staff, treatment protocols are constantly modified and adapted, leading to disparities between institutions and contributing to inconsistencies that may impact the development of resistant bacteria (Miller et al. 2024). There are limited studies focusing on the analysis of antimicrobial practises within facilities offering veterinary care to wildlife, however Miller et al.

(2024) interestingly reported findings which should encourage this sector to reevaluate their practises. The authors found that more than 20% (10/45) of wildlife rehabilitation facilities in the United States (US) used antimicrobials prophylactically, and approximately one third of these organizations did not perform bacterial cultures or antimicrobial sensitivity testing, missing vital information for accurate diagnostics and appropriate targeted treatment. Although this study does not represent all wildlife rehabilitation centres in the US or the rest of the world, these are still relevant results to take into consideration.

Exposure to antibiotics has been shown to alter the gut microbiome through complex microbiota-pathogen interactions, leading to decreased species diversity and ultimately resulting in impaired immune responses and adverse effects on host health. The misuse of these antimicrobial substances has also been associated with different bacterial populations abundant in ARGs (Dongre et al. 2025).

In a study evaluating the effect of antibiotic treatment on gut microbiota in captive lemurs, Bornbusch et al. (2021) reported a rapid reduction of microbial diversity during the treatment phase in animals receiving antibiotic treatment in comparison with those from the control group. Moreover, given the significant differences identified between the two groups for nearly four months post the treatment plan, a long-lasting imbalance in microbial diversity was also associated with antibiotic treatment. On the other hand, ARGs abundance did not consistently correlate with antibiotic treatment history. Animals never exposed to antibiotics showed high levels of resistance genes, while one animal exposed to numerous antibiotic treatments had among the lowest levels observed. These findings hint at the involvement of other factors beyond antibiotic exposure in influencing ARGs abundance, which certain authors consider a marker of antibiotic resistance pollution (Laborda et al. 2022). Consistent with this, Schmartz et al. (2024) identified that free-ranging wildlife harboured resistance genes targeting a narrower range of antimicrobial compound classes than zoo-housed animals, suggesting that increased exposure to human-associated antibiotics and potential transmission pathways in captivity contribute to resistance dissemination.

Besides altering the diversity of the gut microbiome, the use of antibiotics in captive settings introduces opportunities for the selection of ARB (Power et al. 2013). Commensal or opportunistic bacteria that possess intrinsic resistance or acquire ARGs may become dominant within the hosts' gut microbiota (Konstantinidis et al. 2020). Several reports support this hypothesis, highlighting significant increases in AMR in selected bacteria following antibiotic treatment during hospitalization or rehabilitation of wild animals (Ishihara et al. 2012; Fernandes et al. 2024)

While controlled environments such as these provide crucial support to wildlife care, antibiotic stewardship is critical and must be a priority in order to mitigate wildlife-associated public health risks associated with AMR.

2.2.2.3. Diet

Dietary differences between captive and free-roaming wild animals can substantially restructure the gut microbiome, impacting bacterial dynamics and functional pathways, which in turn can contribute to the emergence and persistence of ARGs (Liu et al. 2021; Huang et al. 2025).

Studies on the gut and oral microbiota of captive wildlife identified significant differences in the bacterial composition of animals with varying diets, more specifically herbivores, omnivores and carnivores. In zoos and animal sanctuaries, where the animals' diets are based on human-mediated feeding, this evidence raises questions as to the extent to which captivity alters the microbiota of wildlife (Schmartz et al. 2024). Further, the study by Schmartz et al. (2024) also compared the taxonomic similarity of captive animals with free-ranging wildlife and found notable similarities in the gut microbiota of several zoo-housed species, contrasting with the wildlife analysed. These observations support the idea that nutrition in a controlled environment may have an influence on microbiome composition and that sharing the same living environment might drive gut microbiota of distinct species to converge (Zhou et al. 2022). A more targeted study on the gut microbiota of carnivores kept under different dietary regimes and living environments would allow to better assess how diets in captivity contribute to bacterial diversity, eliminating other impacting variables such as digestive system physiology and dietary composition differences.

Additionally, a study characterizing the AMR of zoo-kept animals found that 94% (17/18) of multi-drug resistant (MDR) *E. coli* strains isolated were from either carnivores or omnivores while 100% of the MDR *Enterococcus faecalis* strains were isolated exclusively from carnivores. These findings suggest that diet, particularly in captivity, may influence and shape not only gut microbial composition and diversity, as supported by Zhou et al. (2022), but also the emergence of resistant bacterial strains. These results are supported by earlier research demonstrating a similar pattern of high prevalence of MDR *E. coli* isolates in carnivores (Bamunusinghage et al. 2022). Both studies explore potential explanations for their results, with the authors suggesting that the carnivores' high trophic level and specific feeding habits may have contributed to the antibiotic resistance observed. Diet has also been detected as a significant factor impacting ARG abundance in captive wildlife in a study focusing on investigating the impact of captivity on the gut microbiome of giant pandas, highlighting the crucial influence of diet in modulating the gut resistome (Cai et al. 2024).

Moreover, the presence of resistant bacteria in animal-derived food products, more specifically raw meat, has been previously demonstrated in earlier papers (Treiber and Beranek-Knauer 2021), raising the possibility that contaminated food may contribute to the

transmission of resistant bacterial strains in captive wildlife through their diet. This concept has also been described as a possible contributing factor to the high levels of ARB detected primarily in carnivores (Medina et al. 2024), reinforcing the role of diet as a potential pathway for resistance transmission. Additionally, Medina et al. (2024) reported a possible association between the presence of Extended-Spectrum Beta-Lactamase (ESBL)-producing *E. coli* in zoo-housed mammals and the diet of carnivorous species, which the authors suggested could be attributed to the use of antibiotics in food-producing animals in Ecuador. These findings highlight the diet of captive animals as a potential route of entry for relevant resistance genes within these environments.

Beyond differences in dietary composition, nutritionally simplified diets commonly implemented in captive environments have also been shown to influence the gut microbiota. As opposed to free-ranging wildlife, which have access to a wide variety of food sources, captive animals often get fed human-formulated diets which lack the complexity of their natural feeding ecology. This reduction in nutrient variety has been previously linked with a decrease in microbial diversity, a pattern observed across multiple species in zoological settings. For example, Clayton et al. (2016) identified simplified commercial diets, lacking in dietary plant fibre, as the primary driver of microbiome perturbation in captive primates. Comparably, diet was also considered by McKenzie et al. (2017) as an important variable associated with primates having the largest changes in gut bacteria in captivity. Similar trends have also been reported in carnivores, with recent studies urging for greater attention to the diets of captive felids (Sun et al. 2025) and herbivores (Zhao et al. 2024) under human care.

These findings also underscore the concern that significant changes in gut microbial communities may lead to dysbiosis, which has been associated with increased susceptibility to infections (Chong et al. 2019). This knowledge supports the hypothesis of a low diversity microbiome providing an environment in which pathogenic organisms encounter reduced microbial competition and therefore facilitates pathogenic growth and the potential to develop resistance to antibiotics (Dongre et al. 2025). This can be applied to captive settings where, as previously mentioned, the above factors contribute, individually and in combination, to reduced microbial diversity. However, not all literature supports this perspective. Contradictory evidence exists suggesting that diet provided to captive wildlife can sometimes be of a higher nutritional value, owing to consistency and more abundant food sources (Zhou et al. 2022). Furthermore, the resilience of an animals' gut microbiota is strongly influenced by their diet composition and quality which, if effectively replicated in human-managed environments, can be maintained relatively unperturbed by aspects of captivity (Bornbusch et al. 2021), potentially reducing the selection pressures that drive the emergence and persistence of ARB.

2.3 Non-domestic Felids as Reservoirs of Antimicrobial Resistance

The conservation of non-domestic felids is critical to biodiversity, yet many species are increasingly impacted by anthropogenic pressures (Thompson et al. 2021). With around 50% of the species included in this taxonomic family being considered threatened according to the IUCN Red List (IUCN 2025), conservation initiatives have increased their focus in developing captive breeding programs in support of population recovery. Unintentionally, this elevates the risks of bidirectional transmission of zoonotic pathogens, including ARB, a trend that has been documented in scientific literature characterizing AMR among felids in zoological settings (De Witte et al. 2021). Large felids are often considered popular species attracting the public and institutional attention. For that reason, they are commonly found in zoological collections and frequently targeted in captive wildlife research, making them ideal sentinels of resistant bacterial pathogens in worldwide disease surveillance systems (Rampacci et al. 2024).

While big cats can be valued as early-warning indicators of environmental AMR reflecting exposure levels in wildlife surveillance, they are predominantly viewed as reservoir species that actively harbour and amplify resistant bacterial strains in primary AMR research focusing on captive conditions. Several studies on species including lions, tigers and jaguars support the role of non-domestic felids as reservoirs for AMR dissemination, underscoring their significance in the persistence and spill-over of resistant bacteria within zoological settings (Ghosh et al. 2019; Medina et al. 2024; Rampacci et al. 2024). For example, clonally related ESBL-producing *E. coli* strains were previously isolated from two zoos in Belgium, from an Amur tiger, an Amur leopard and a Spectacled bear in Zoo 1 and from a Spotted hyena and an African lion in Zoo 2. These findings are suggestive that non-domestic felids, alongside other carnivores, may act as important reservoirs of ARB and, as hypothesised by De Witte et al. (2021), may indicate possible clonal expansion of *E. coli* strains. The recurrence of the same strain across taxonomically diverse animal species highlights not only the role of zoological parks in interspecies transmission but reinforces the role of captive wild felids in AMR dynamics.

Further evidence of AMR in non-domestic felids is highlighted by Kim, Kim, et al. (2024), who have reported MDR strains in Amur leopard cats. This study also indicates the potential expansion and dissemination of *E. coli* strains within the captive environment following the acquisition of MDR, as evidenced by 17 MDR isolates from different species clustering within the same phylogenetic clade. The isolation of MDR *E. coli* strains within wild felids, namely captive Amur tigers, has been documented earlier in China by Xue et al. (2013) where it is also demonstrated that these animals may act as potential disseminators of resistance genes. This study identified class 1 integrons containing resistance genes, and multiple associated

resistance determinants, underlining the frequency of emergent resistance in these species and their broader implications for the management of wildlife populations, particularly considering the potential for horizontal gene transfer (HGT) to facilitate the dissemination of ARB in non-domestic big cats in captivity.

Recent research has also detected resistant *E. coli* in wild populations of Amur tigers and leopards in China, revealing a diverse range of ARGs alongside high rates of virulence determinants. These findings reinforce how free-ranging big cats, as well as their captive counterparts, contribute to AMR dissemination and act as significant reservoirs of resistance, highlighting the importance of these endangered species in surveillance programs (Li, Lan, et al. 2024).

Moreover, as apex predators with long lifespans, wild felids in captivity are subjected to frequent human monitoring, placing them at a high risk of exposure to ARB (Rampacci et al. 2024). This exposure can occur through several routes, as previously described. Potential transmission routes include their diet, specifically through the consumption of raw meat, contact with contaminated environments and indirect transfer from zookeepers and veterinary staff, which reflect the broader aspect of AMR circulation that may occur within and beyond zoological settings (Vittecoq et al. 2016; Laborda et al. 2022). Besides potential zoonotic transmission to humans, the spread of ARB or ARGs between individuals of the same or different animal species through shared environments is often exacerbated in zoos. This risk is elevated by common zoo management practises, including the rotation of animals between enclosures as part of *ex-situ* breeding programs and the relocation of same-species individuals into different zoological parks as part of managed metapopulations initiatives across institutions (De Witte et al. 2021). Such practises may facilitate AMR dissemination and inadvertently pose a risk not only to a variety of species housed in the same institution but also to occupationally exposed people. These dynamics highlight once again the link to One Health principles in the structuring of wildlife management strategies for AMR monitoring across conservation organisations.

2.4 Antimicrobial Stewardship in Wildlife Conservation

While the public health risks of AMR in zoological institutions have been previously explored, targeted stewardship practices remain an underdeveloped and under-researched yet crucial pillar of AMR mitigation strategies within wildlife conservation (Miller et al. 2024). Currently, there is very limited direct information focusing on this topic in captive wild animals,

exposing the need for defined species-specific and context-specific protocols applicable in wildlife conservation settings.

2.4.1 Role and Importance of Antimicrobial Stewardship

The term antimicrobial stewardship (AMS) was established in the 1990s, where it was first used in a published article written by American authors (McGowan and Gerding 1996). Since then, this term has evolved in different settings, influenced by various interpretations depending on the country and the disciplinary background of professionals which has resulted in multiple definitions. Dyar et al. (2017) defines antimicrobial stewardship as 'A coherent set of actions which promote using antimicrobials responsibly'. The widespread application of this concept has led to reports aiming at defining 'responsible antibiotic use' globally to guide stewardship activities (Monnier et al. 2018).

The use of antimicrobials in veterinary medicine represents a key target for intervention within antimicrobial stewardship programs. Internationally, regulations have been developed by recognised organisations, including the WHO's Medically Important Antimicrobials (MIA) List (World Health Organisation 2024), to restrict the use of high-priority antibiotics in veterinary medicine and thereby safeguarding their effectiveness for human healthcare (Alhassan et al. 2025). This is a relevant example of measures implemented within a stewardship program, strengthening the broader concept of antimicrobial management which accounts not only for the immediate impact at an individual level but also for the long-term preservation of effective therapies and the growing recognition of the interdependence between human and animal health (Dyar et al. 2017). Nowadays, these strategies are a cornerstone of One Health programs to combat AMR, with leading international organisations emphasizing the interconnected stewardship responsibilities shared by human and veterinary healthcare as well as environmental management (World Health Organization et al. 2022).

With the knowledge that antibiotic use is the main driver of AMR, addressing this concern through veterinary AMS is fundamental for the protection of public health on a global scale, since around 70% of antibiotics sold worldwide are exclusively used in livestock production (Alhassan et al. 2025). Effective AMS programs applied to livestock have been demonstrated to successfully reduce the use of antibiotics in countries such as Denmark and the Netherlands (Levy 2014; Speksnijder et al. 2015), showcasing the importance of reinforcing evidence-based policies that prioritize surveillance and responsible veterinary antibiotic prescriptions. Finland and Norway are also amongst the Nordic countries with the lowest veterinary antibiotic usage in Europe post longstanding AMS practises (Sternberg-Lewerin et al. 2022).

As scientific evidence documenting the presence of clinically important resistant pathogens and their resistomes in wild mammals, birds and reptiles worldwide continues to increase over time (Li et al. 2024), the implementation of AMS principles must be extended to wildlife conservation settings. This includes improving the diagnostic capabilities and veterinary prescribing practises within captive wildlife facilities. While species-specific guidelines regarding antimicrobial agents of veterinary importance are mentioned to be under development for food-producing animals such as poultry, swine and aquatic species (Ferreira et al. 2022), expansion of such targeted practises to cover a wider range of wildlife species remains notably absent.

2.4.2 Veterinary Prescribing Practises in Zoos, Wildlife Rehabilitation Centres and Exotic Pets

As a result of the exponential use of antibiotics worldwide, it was necessary to define the term ‘responsible use of antibiotics’ and translate it into clear and distinct actions to be promoted across human and animal health (Monnier et al. 2018; Ferreira et al. 2022). However, although AMS in wildlife should be based on the same principles as in other sectors of veterinary medicine, formal guidelines have, as of yet, not been adapted to conservation contexts (Miller et al. 2024), leading to the off-label use of antibiotics to be a common practise (Broens and van Geijlswijk 2018).

Amoxicillin-clavulanate and fluoroquinolones, particularly enrofloxacin, are among the antibiotics most frequently described in the treatment of zoo-housed wild species, rehabilitated wildlife and exotic pets (Barbosa et al. 2023; Sealey et al. 2023; Miller et al. 2024). In a retrospective study analysing antimicrobial prescriptions in exotic pets and wildlife at a veterinary teaching hospital in Spain, in which birds are the most common animal group, marbofloxacin was reported as the most commonly prescribed antibiotic (Romero et al. 2024). These findings are consistent with those from Hösli et al. (2021), who identified fluoroquinolones as the most frequently used antibiotics among Swiss veterinarians treating exotic pets. Collectively, these studies represent an essential first step for using evidence-based prescribing patterns as a foundation for the development of formal antibiotic stewardship guidelines tailored to non-traditional companion animals, potentially offering guidance to zoo-housed species and wildlife under rehabilitation care. Moreover, they underscore the broader importance of collecting antibiotic usage data to support informed decision-making in wildlife health management.

Across wildlife and exotic animal care settings, several patterns emerge that highlight stewardship concerns, one of them being the frequent use of antimicrobial agents classified

as critically important for human medicine (EMA, 2019). For instance, Romero et al. (2024) reported the recurrent use of category B (Restrict) marbofloxacin in their study performed in Spain. Notably, the use of category D (Prudence) antibiotics which are recommended as first-line options, when possible, was found to be relatively low in the same study. These findings raise concerns in regard to the potential of cross-species transmission of resistant pathogens, which can lead to compromised efficacy of treatments in human medicine and contribute to the worldwide dissemination of AMR.

Another significant stewardship concern is the prophylactic administration of antibiotics, often without evidence of infection. While perioperative prophylaxis remains an advised practise in certain high-risk surgical cases, authors highlight the limited research available concerning wildlife compared with domestic species (Fiorello et al. 2016). Species-specific recommendations from the Association of Primate Veterinarians (2021) emphasize a more cautious approach. Guidelines on captive primate management indicate that prophylactic antimicrobial use should not be considered a preventative health strategy and states that the use of postoperative antimicrobials is considered unnecessary for the majority of surgeries to be performed in these species. These guidelines emphasize the importance of limiting antimicrobial therapy to objectively justified cases in order to decrease resistance pressures. These recommendations are supported by European Union (EU) law on veterinary medicinal products restricting the prophylactic use of antimicrobials, described in the Council Regulation 2019/6 of the 11 December 2018 (European Parliament and Council 2019).

The empirical use of antibiotics without diagnostic confirmation represents a further area of concern. A study in Brazil reported that the treatment of exotic pets with antimicrobials is frequently performed without the confirmation of the etiological agent, whether through molecular techniques, culture or antibiogram (Barbosa et al. 2023). This practise contradicts international recommendations, such as those from the British Small Animal Veterinary Association (BSAVA 2022), which restrict the use of fluoroquinolones, mentioned are the most commonly used agent in this study, to cases supported by results from culture and sensitivity testing. The reliance on broad-spectrum antibiotics and the limited use of diagnostic testing in clinical decision-making hinder AMS efforts and highlight the constraints faced in wildlife and exotic animal medicine.

To the best of the author's knowledge, Miller et al. (2024) remains the only large-scale survey of veterinary antimicrobial practises in wildlife rehabilitation settings. While several microbiological studies document AMR prevalence in zoos, there is a surprising absence of research on antimicrobial use (AMU) policies, stewardship guidelines or prescribing behaviours in zoological medicine settings. This observation highlights an important gap in existing literature and a key area for future stewardship-focused research. Spanish authors support this same opinion, observing that veterinary clinicians often rely on 'personal

experience of successful strategies' to guide their chosen treatment plan due to a lack of species-specific protocols (Romero et al. 2024). The British Veterinary Association's (BVA) past president, Liz Mullineaux, shares the opinion that the overuse of antibiotics is 'huge' in the wildlife rehabilitation sector (Mullineaux 2023).

However, wildlife health cannot rest solely on veterinary professionals and requires responsibility to be divided between sectors, including the pharmaceutical industry and food animal producers, through an interdisciplinary collaboration between institutions (Goulet et al. 2024). Equally important, the findings presented above underline the pressing need for real-world data to guide antimicrobial prescriptions in less commonly treated species. Generating such evidence is crucial for informing AMU protocols that integrate AMR concerns and strengthen stewardship practises within conservation medicine (Jelinski et al. 2022).

2.4.3 Challenges for Antimicrobial Stewardship Implementation in Captive Wildlife

According to Dyar et al. (2017), accurate diagnostics, compliance with antimicrobial guidelines and reassessing treatment necessity are essential for the responsible use of antimicrobials. In theory, these actions could directly influence the behaviour of medical professionals; however, in settings such as captive wildlife facilities these strategies may be hard to implement and therefore become a challenge for veterinary staff working in these organisations.

Wildlife and zoo veterinarians often encounter significant barriers which complicate the implementation of AMS. One of the most recognised challenges is the limited or inadequate diagnostic options available for wildlife health evaluation. Ryser-Degiorgis (2013) reported that wildlife health investigations often have to rely on domestic animal diagnostics due to the lack of specific tests for wild animals and stresses the difficulties associated with wildlife-specific validation of diagnostics tools. As a result, inadequate diagnostic capacity often leads to empirical treatment, heightening the likelihood of inappropriate antibiotic use (Drane et al. 2021).

In addition to the species-specific knowledge required in wildlife medicine, such as species' anatomy, pathology and disease susceptibility, the lack of pharmacokinetic and pharmacodynamic (PK/PD) data for wild species presents another challenge to the development of AMS protocols. Evidence-based dosing regimens are essential for therapeutic efficacy and to minimize resistance selection (Caneschi et al. 2023), however wildlife veterinary dosing guidelines are often extrapolated from domestic species. This approach could potentially result in species-specific subtherapeutic or toxic outcomes due to

physiological and metabolic differences (Barbosa et al. 2023). Similarly, Jelinski et al. (2022) identified the lack of PK/PD data as a critical limitation in the development of antibiotic treatment recommendations for exotic species, complicating efforts to promote safe and effective AMU. This absence of readily accessible and validated dosing information not only compromises animal welfare but also impacts efforts to standardize AMU across facilities involved in the care of wild animal species.

Another important obstacle to AMS implementation in wildlife settings is the absence of formal data collection systems for AMU and AMR. Efficient surveillance systems are essential for establishing standardized antimicrobial usage patterns, detecting emerging resistance trends and guiding medical prescribing practises. In institutions working with wildlife, exotic pets, and zoo-housed species, the lack of coordinated data systems hinders the possibility of assessing AMR risks (Vercelli et al. 2022). Although global databases such as ZIMS provide shared access to aggregated information, including drug dosage data and clinical reference values, this platform is primarily designed to support clinical record management rather than coordinated AMR surveillance. As such, while they facilitate general clinical knowledge exchange, the absence of a standardized system for recording and comparing data relevant to AMU and AMR across institutions continues to limit progress, particularly for under-studied species. This challenge is well expressed by Goulet et al. (2024), noting that in the absence of an international consensus, individual countries are left to independently navigate the complexities of wildlife health management. The authors further advocate for the development of wildlife health policies and interconnected legislature, proposing an operational framework to facilitate the integration of wildlife into existing animal and human health regulations.

This issue is further intensified by the lack of studies documenting treatment failures in wildlife care facilities, including the inadequacy of animal release due to the presence of resistance infections. This notable gap in the literature highlights another crucial area for future research, as understanding the clinical outcomes of AMR in these settings is essential to inform conservation decisions and veterinary care. Given the widely reported AMR findings in wild animal species, the absence of such data limits the ability to assess the impact of resistant infections and, once again, limits progress towards targeted AMS protocols tailored to wildlife (Benavides et al. 2024).

Despite these persistent challenges, recent regulatory developments offer potential opportunities for the progression of AMS implementation across wildlife settings. Within the EU, recent regulations permit member states to progressively extend the collection of AMU data to non-food-producing animals by no later than 2030, described in the EU Regulation 2019/6 of 11 December 2018 (European Parliament and Council 2019). Such policies could serve as a potential incentive for expanding existing surveillance systems such as the

European Antimicrobial Resistance Surveillance Network in Veterinary Medicine (EARS-Vet) and the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) to include wildlife, zoo-housed and exotic species allowing for the evaluation of policy adherence and real-time alterations of stewardship strategies across a broader spectrum of animal species (Lagrange et al. 2023; Ching et al. 2024; Romero et al. 2024).

In parallel, global networks such as the Species Survival Plans (SSPs) developed by the Association of Zoos and Aquariums (AZA) and EAZA *Ex-situ* Programmes (EEPs) offer a potential platform for continued tracking of AMR and data sharing across zoological institutions. Utilizing these existing frameworks for AMR and AMU data collection could greatly enhance antimicrobial management strategies in these facilities, serving as a step in the right direction for coordinated AMR surveillance efforts (Benavides et al. 2024).

2.5 *Escherichia coli* in Veterinary Medicine

Escherichia coli is a facultative anaerobic, Gram-negative bacterium that exists both as a commensal, mainly in the lower gastrointestinal tract of mammals (Lupindu 2017), and as an opportunistic pathogen responsible for a wide spectrum of infections across species (Nielsen et al. 2022). In veterinary medicine, it is a frequently documented etiological agent of post-weaning diarrhoea in piglets (Paiva et al. 2025), urinary tract infections in companion animals (Jousserand et al. 2025), and septicaemia in both livestock and captive wild species (Gu et al. 2025; Zhang et al. 2025).

In addition to its impact on animal health, *E. coli* has also been recognized for its zoonotic potential, particularly of toxin-producing strains. De Luca et al. (2025) reported such strains in domestic species, while Chowdhury et al. (2025) identified Shiga toxin-producing *Escherichia coli* (STEC) -positive isolates in dairy cattle, which pose direct risks to human health. These findings represent an effective application of the One Health Approach and of the management of shared threats across species.

2.5.1 Ongoing Significance and Pathogenic Potential of *E. coli*

Escherichia coli continues to be a pathogen of significant relevance to the present day, posing a threat to a wide host range across numerous animal species, as exemplified by recent public health incidents such as the detection of *E. coli* in the water supply at Belfast Zoo in June 2025 (Cochrane 2025). Current research further emphasizes the pathogen's continued significance, particularly when it comes to zoonotic strains. For example, recent surveillance

on the occurrence of STEC in zoo animals was confirmed in Pakistan (Rasheed et al. 2023) as well as in free-ranging deer in Poland (Szczerba-Turek et al. 2023), raising awareness on the silent circulation of pathogenic *E. coli* strains in distinct and disperse environments.

A compelling example of the pathogenic potential of *E. coli* lies in the evolution and persistence of MDR strains detected across diverse animal hosts. The emergence and intensification of resistance in this species has been disclosed by Tadesse et al. (2012), who reported a significant upward trend in MDR *E. coli* isolates from animals since the 1950s. More recent studies reinforce this pattern, including the report of a high prevalence of MDR *E. coli* in companion animals in China (Teng et al. 2023). These findings are notably observed regardless of antibiotic use and suggest a persistent transmission and maintenance of such strains within pet populations. The impact on livestock has also been heavily acknowledged, including in chicken farms in Uganda (Nyolimati et al. 2025), reflecting the consequences of widespread antibiotics use in food production. Wildlife species including birds and various terrestrial mammal species, as mentioned previously, have also been shown to harbour MDR strains (Sabença et al. 2024). Collectively, these results underline not only the bacterium's widespread distribution and adaptability but also its capacity to persist as a reservoir of resistance genes. In some studies, these MDR strains have also been found to carry important virulence factors, highlighting their ongoing threat to public health on top of individual animal health (Lee et al. 2024).

The pathogenic potential of *E. coli* is closely associated with its capacity to express a wide variety of virulence factors, including adhesins, hemolysins, siderophores and toxins (Sora et al. 2021), contributing to its ability to colonize, invade and cause disease in animal hosts. In veterinary medicine, multiple studies have identified *E. coli* strains harbouring a mix of virulence genes, often representing different pathotypes (Bertelloni et al. 2020; Zhao et al. 2021; Oyaba Yinda et al. 2022; Feitosa et al. 2024; Nammuang et al. 2024). Moreover, some studies have also revealed similarities in virulence gene profiles between isolates from animals and humans, suggesting potential cross-species transmission and reinforcing animal species, particularly wildlife, as reservoirs of pathogenic *E. coli* (Murphy et al. 2021; Pista et al. 2022). Furthermore, the co-occurrence of virulence genes alongside AMR in *E. coli* animal isolates emphasizes the potential for a variety of animal species to harbour therapeutically challenging pathogenic strains. As such, *E. coli* continues to cross boundaries between species and environments, and due to its broad host range and established zoonotic potential, the urgency for targeted surveillance increases (Di Marcantonio et al. 2025).

Characterizing wildlife-associated *E. coli* remains essential to understand disease risk and anticipate resistance dissemination patterns, particularly as human-animal interfaces continue to expand (Lagerstrom and Hadly 2021).

2.5.2 *E. coli* as an indicator of AMR

Beyond its clinical significance, *E. coli* has emerged as a key indicator bacterium for AMR surveillance programs in both domestic and wild animal populations, as well as in the environmental sector. Its wide distribution, ability to acquire and disseminate resistance genes, well-established methods of isolation and well-recognised role as a marker of anthropogenic influence make it an ideal sentinel for the monitoring of AMR trends as part of One Health surveillance efforts (Anjum et al. 2021; Furuya et al. 2022).

In wildlife surveillance, *E. coli* is consistently reported as one of the most frequently isolated resistant organisms, alongside *Salmonella* spp. and *Enterococus* spp. (Vittecoq et al. 2016). Its detection across a diverse range of species and environmental settings allow for comparative analysis between a spectrum of AMR studies, including free-ranging and captive wildlife research (Li, Mowlaboccus, et al. 2024). In addition to its relevance to wildlife, *E. coli* has also been identified among the most relevant organisms for resistance monitoring in livestock and companion animals (Nielsen et al. 2022).

The significance of *E. coli* also lies in its inclusion across all three AMR categories proposed by Caneschi et al. (2023): AMR in host-adapted pathogens, AMR in zoonotic agents and AMR within commensal bacterial populations. Among these categories, resistant *E. coli* within commensal microbiota has been mentioned to represent the most important threat due its overwhelming abundance in animal and human hosts. These bacterial populations, serving as dominant reservoirs for ARGs, create opportunities for HGT to potentially more pathogenic bacteria within animal hosts or in the environment (Tawfick et al. 2022).

From a broader perspective, *E. coli* plays a central role in understanding the mechanisms and pathways involved in the dissemination of AMR and it continues to be a focal point of extensive scientific research in this field encompassing a variety of primary reservoirs (Di Marcantonio et al. 2025).

Section 3. Study Objectives

Despite the existing research on antimicrobial resistance (AMR) in wildlife, a limited understanding remains on how captivity shapes the microbial profiles of wildlife under human care, and how such changes may carry implications for animal health, conservation efforts, and potential zoonotic occupational risk.

The purpose of this study was to investigate the prevalence and patterns of antimicrobial resistance and virulence phenotypic factors in *E. coli* isolates obtained from a variety of captive non-domestic felid species. This study also aimed to compare these profiles

between individuals and distinct species within the Felidae family in order to evaluate the role captivity plays in shaping bacterial resistance and pathogenicity. To achieve this, independent variables related to captivity and individual animal characteristics were statistically analysed for their potential influence.

This study aims to contribute an additional perspective to the existing literature by providing insights specific to non-domestic felid species and by addressing captivity as a determinant factor of AMR.

Section 4. Materials and Methods

4.1 Sample Collection

The faecal samples used in this study were collected in a conservation centre, focused on the protection of non-domestic felids, in the south-east of England. A total of 41 samples were included in this study, including duplicates, collected from captive animals belonging to 11 animal species. The samples obtained included 5 composite samples, obtained from enclosures housing more than one animal, and 36 individual samples. Composite faecal sampling was used when an individual sample could not be associated with absolute confidence to one specific animal within an enclosure.

A total of 36 captive felids were included in this study, having been relocated to live in the centre for periods ranging from 19 years to a little over a year. Relocations were due to several reasons, and include transfers from other European zoos, rescue projects, private collections and circuses. Nine felids were included in the 5 composite samples collected, comprising of 3 separate species pools. The first pool included 2 samples from a lioness pride of 4; the second included 2 samples from a snow leopard mother and 2 cubs and the third included 1 sample from 2 adult cheetahs. Regarding the inclusion criteria for the sampled population, all animals selected for this study were born in captivity and resided permanently at the sanctuary during the sample collection period. In this facility, all felids were housed in earthen-floored enclosures with year-round access to an outdoor display area as well as an indoor area and were fed a diet that mainly consisted of raw horsemeat, venison and chicken.

Samples were obtained during a 4-day period, from 5/09/2024 to 8/09/2024, according to the weekly schedules for feeding and cleaning of the cats' enclosures, allowing access to the indoor or outdoor areas. AMIES transport swabs (VWR®, Leuven, Belgium) were used to collect the faecal samples located in the enclosures once the respective cats were securely moved.

From the perspective of animal ethics, all data collected was acquired through non-invasive methods and there was no deviation from the animals' normal husbandry routine.

A fresh sample, defined in this study as deposited within the previous 24 hours, was selected for sampling when available. The faecal matter was divided in half using a knife sterilized with 70% ethyl alcohol, after which a sterile swab was inserted approximately 2cm in the centre of the faecal sample. Each swab was gently rotated to ensure that a visible coat of faecal matter was collected. Immediately after, each swab was stored in the respective tube for adequate transport and kept at 4°C until transportation to the Laboratory of Microbiology and Immunology at the Faculty of Veterinary Medicine at the University of Lisbon, Portugal, for further processing.

A brief clinical history was also collected in regard to each sampled animal, to gather information on the animals' sex, age, origin, time at the sanctuary, previous treatments, vaccinations and deworming, diagnosed illnesses, IUCN status and proximity with humans. Data regarding 'proximity with humans' was categorized according to the presence of keepers within the enclosures with the animals present and frequency of the animals' involvement with the public, taking into consideration various animal activities for example 'keeper for the day' and 'big cat encounters'. The experience 'keeper for the day', involved members of the public aiding the animal keepers with the daily upkeep of the enclosures, associated with an increased human presence within the animal's permanent residence. 'Big cat encounters' involved the public getting up close and feeding the animals through wire mesh panels surrounding the enclosures, included as an opportunity for increased human-animal proximity. The organization of this data into categories can be found in Table 1.

Table 1. Data categories included within the human proximity factor.

Human proximity factor				
Keeper in enclosure with animal present	Keeper in enclosure without animal present	Human-Animal Interaction		
		Direct	'Keeper for the day'	'Big cat encounter'

4.2 *E. coli* Isolation and Identification

Each sample was inoculated in MacConkey agar medium (VWR®, Leuven, Belgium) and incubated at 37°C for 24 hours. This medium is commonly used for the isolation of Gram-negative enteric bacteria and allows the differentiation between lactose fermenting and non-fermenting Gram-negative bacteria. The presence of selective agents, crystal violet and bile salts prevent the growth of Gram-positive bacteria and fastidious Gram-negative bacteria (Jobin et al. 2024). The typical appearance of lactose-fermenting colonies is pink or red, while

those of non-lactose-fermenters are colourless. *E. coli*, unlike other weaker lactose fermenters, can strongly ferment lactose to produce sufficient acid to result in the precipitation of the bile salts present in the medium, creating a pink halo around individual colonies or areas of confluent growth in the agar (Harrigan and McCance 1966; Allen 2016).

Each MacConkey plate was observed to evaluate the macroscopic morphology of the bacterial growth. The lactose fermenting pink, round, medium-sized colonies surrounded by a visible pink halo, compatible with the precipitation of bile salts, were identified as *E. coli* presumptive isolates (Lupindu 2017; Allen 2016). After, a minimum of 4 colonies with distinct macroscopic morphology were selected from each plate and identified as A, B, C or D. Morphological characteristics used for differentiation between colonies included mucoid or dry texture, umbonate, crateriform or flat morphology, and colour shade (pale vs dark pink colony). Presumptive *E. coli* colonies were reisolated in MacConkey agar and incubated at 37°C for 24 hours aiming to obtain pure cultures. Next, the isolates obtained were inoculated onto Brain Heart Infusion (BHI) agar (VWR®, Leuven, Belgium) and incubated at 37°C for 24 hours, allowing to obtain a fresh culture to perform Gram staining for microscopic observation and the oxidase test, aiming to identify Gram-negative, oxidase-negative bacilli (Hossain et al. 2021). The isolates that matched this characterization were stored in buffered peptone water (VWR®, Leuven, Belgium) with 20% glycerol (VWR®, Leuven, Belgium) at -20°C until further testing.

The identification of presumptive *E. coli* isolates was confirmed by IMViC testing (Indole, Methyl Red, Voges-Proskauer, Citrate), through inoculation in three culture mediums: Simmons Citrate agar, Sulphide Indole Motility (SIM) agar and Methyl Red Voges-Proskauer (MRVP) medium. This primary biochemical test series is widely used for the differentiation and identification of members of the *Enterobacteriaceae* family (Dahal 2022).

MRVP (Oxoid™, Hants, UK) medium allows for the execution of 2 independent biochemical tests used to determine different types of glucose fermentation. Each isolate was inoculated in two tubes of MR-VP broth, incubated at 37°C for 24 hours. The Methyl Red (MR) test detects mixed acid fermentation performed by some bacteria as their preferred glucose metabolic pathway. After incubation, mixed acid fermenters develop a red ring atop the superior layers of the medium once the methyl red indicator is added, indicating a positive result. The Voges-Proskauer (VP) test detects the bacteria's ability to metabolize pyruvate into 2,3-butanediol via the butanediol fermentation pathway. In this test, a red ring appears in case of a positive result following the addition of reagents VP1 and VP2 (bioMérieux© SA, Marcy l'Étoile, France).

Simmons Citrate agar (Oxoid™, Hampshire, UK) was used to test if an organism can survive using citrate as the sole source of carbon. After inoculation and incubation at 37°C for 24 hours, a citrate positive species is identified through a colour change from green (neutral

pH) to blue (alkaline pH), associated with the presence of the citrate permease enzyme which transports citrate into the cell, resulting in the production of alkaline byproducts.

Sulphide Indole Motility (SIM) (Oxoid™, Hampshire, UK) agar provides information on 3 bacterial properties: motility, indole production and H_2S production. After stab inoculation and incubation at 37°C for 24 hours, positive motility is observed by the appearance of a cloudy growth around the stab, corresponding to the ability of the bacteria to disperse away from the inoculation line. H_2S production is demonstrated by black coloration of the medium, while indole production is detected with the addition of Kovac's Reagent (Merck KGaA, Darmstadt, Germany). If an organism produces the enzyme tryptophanase, the formation of a red ring is observed at the top of the medium indicating the presence of an indole positive bacterial species.

These biochemical tests allow to identify *Escherichia coli* isolates as those presenting positive results in both the indole and methyl red tests, and negative results in the hydrogen sulphide production, citrate utilization and Voges-Proskauer tests (Hossain et al. 2021 Jul 11; López-Islas et al. 2022; Fernandes et al. 2024; Mall et al. 2024). Both motile and non-motile *E. coli* isolates have been reported in clinical and environmental studies, therefore in this study motility was recorded but was not a form of isolate exclusion (Andrade et al. 2002; Ma et al. 2021).

Due to the collection of duplicate samples, potentially resulting in the duplication of isolates, these were re-isolated on MacConkey agar and their morphology compared. From these, morphologically similar isolates were excluded, and only morphologically distinct isolates were subjected to further characterization.

In the cases where isolates revealed IMViC test results inconsistent with the typical *E. coli* profile, particularly those which were indole-negative, these were retested using the IMViC series and inoculated onto Triple Sugar Iron (TSI) agar for identification confirmation before exclusion from this study.

4.3 Antimicrobial Susceptibility Testing

The evaluation of the isolates' antimicrobial susceptibility profile was performed using the Kirby-Bauer disk diffusion test, according to the Clinical Laboratory Standards Institute guidelines (CLSI 2024a; 2024b).

Briefly, *E. coli* isolates were incubated overnight, and a bacterial suspension was prepared and adjusted to a 0.5 McFarland turbidity. The suspension was then inoculated over

the surface of Mueller–Hinton agar plates (Oxoid™, Hampshire, UK), after which antimicrobial disks were placed onto the agar's surface at equal intervals.

The susceptibility profile of the *E. coli* isolates was assessed using a total of 12 antibiotics (Oxoid™, Hampshire, UK) from 10 distinct classes: beta-lactam with beta-lactamase inhibitor (amoxicillin-clavulanate, AMC, 30 µg), third generation cephalosporins (ceftazidime, CAZ, 30µg and cefotaxime, CTX, 30µg), cephamycins (cefoxitin, FOX, 30µg), fluoroquinolones (enrofloxacin, ENR, 5µg), aminoglycosides (gentamicin, CN, 10µg), monobactams (aztreonam, ATM, 30µg), folate pathway inhibitors (sulfamethoxazole-trimethoprim, SXT, 25µg), phenicols (chloramphenicol, C, 30µg), penicillins (ampicillin, AMP, 10µg), tetracyclines (tetracycline, TE, 30µg) and carbapenems (imipenem, IPM, 10µg). These compounds were selected based on their use in veterinary and human medicine and on the antimicrobial therapy protocols adopted at the conservation centre where the sampling took place. This selection was also based on previous scientific studies with a focus on wild or captive non-domestic felids (Gonçalves et al. 2013; Xue et al. 2013; Li et al. 2024).

The reference strains *E. coli* ATCC® 25922 and *Pseudomonas aeruginosa* ATCC® 27853 were used for quality control. After incubation, the diameter (mm) of the inhibition zone was measured for each compound used, allowing the classification of each isolate as susceptible (S), intermediate (I) or resistant (R) according to CLSI guidelines (CLSI 2024a; 2024b). Randomly selected isolates were used to perform 10% replicas.

Isolates were classified as multi-drug resistant (MDR), defined as non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al. 2012)

4.4 Virulence Profiles

The isolates' phenotypic virulence profiles were determined by assessing their ability to produce certain enzymes associated with bacterial pathogenicity, using differential mediums. In this study, the isolates were tested for their ability to produce protease, DNase, gelatinase, lecithinase, hemolysins and to form biofilm. The isolates were inoculated in BHI agar at 37ºC overnight prior to being inoculated in the testing media. A 10% replica was executed for each test performed with randomly selected isolates.

Biofilm production was assessed using Congo Red agar composed of Brain Heart Infusion broth (VWR®, Leuven, Belgium), bacteriological agar (VWR®, Leuven, Belgium), sucrose (5%) (Sigma®, Darmstadt, Germany) and Congo Red reagent (0.08%) (Sigma®, St Louis, USA). The results were read following incubation at 37ºC for 72 hours. A positive result corresponded to the development of black colonies with a dry crystalline consistency, as

shown in Figure 1. In the case of non-biofilm producers, smooth orange to red coloured colonies were observed (Lee et al. 2016). In this assay, the strains *Enterococcus hirae* ATCC® 10541 and *E. coli* ATCC® 25922 were used as positive and negative controls, respectively.

The DNase test was used to determine the bacteria's ability to produce the deoxyribonuclease enzyme responsible for the hydrolysis of phosphodiester bonds in DNA molecules (Lauková et al. 2020). This test was performed using DNase agar (Remel™, Kansas, USA), incubated for 48 hours at 37ºC. A positive result was revealed by the development of a clear zone around the bacterial growth after the addition of 2ml of 1N hydrochloric acid solution to each plate, as shown in Figure 2. The observation of an opaque medium throughout the plate corresponds to an unhydrolyzed DNA precipitate compatible with a negative result (Pokhrel 2015). *Staphylococcus aureus* ATCC® 25923 and *E. coli* ATCC® 25922 were used as positive and negative controls, respectively.

Figure 1. Biofilm production assessed on Congo Red Agar. Biofilm-positive isolates are seen as darker or black colonies (black arrows).

(original, 2024)

Figure 2. DNase production assessed on DNase Agar. Positive control, *S. aureus* ATCC® 25923 (black arrow), seen surrounded by a clear zone, and DNAse-negative isolates.

(original, 2024)

The gelatin hydrolysis test was used to detect the bacteria's gelatinase activity. Gelatinase is an enzyme secreted extracellularly, depending on the bacterial species, that hydrolyses or digests gelatin (Megur et al. 2023). The production of gelatinase was tested using tubes with 3ml of nutrient gelatin medium (Oxoid™, Hants, UK), incubated at 37ºC for 72 hours. Subsequently, the tube was placed in an ice bath for 15 minutes to determine liquefaction, with a positive result being the presence of liquid medium while in a negative result the medium will remain solid, as shown in Figure 3 (Dela Cruz et al. 2016). *P. aeruginosa* ATCC® Z25.1 and *E. coli* ATCC® 25922 were used as positive and negative controls, respectively.

The production of hemolysins was evaluated using Columbia agar containing 5% sheep blood (bioMérieux© SA, Marcy l'Étoile, France), incubated at 37ºC for 24 hours. Blood agar is an enriched medium that supports the growth of fastidious organisms and is used to

characterise bacteria based on their hemolytic properties (Buxton 2016). The production of extracellular hemolysins by bacteria causes the lysis of red blood cells, as such, the type of hemolysis was detected through the observation of the zone surrounding the bacterial growth. Beta hemolysis is defined by a clear halo around the colonies as a result of complete lysis of the red blood cells present in the medium, while alpha hemolysis results in partial lysis, resulting in a greenish-brown discoloration around the colonies. A non-haemolytic presents no discoloration or hemolysis zone visible under or around the colonies, as shown in Figure 4. *S. aureus* ATCC® 25923 and *E. faecium* CCUG 36804 were used as the positive and negative controls, respectively.

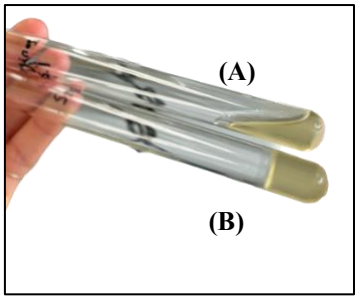


Figure 3. Gelatin hydrolysis test post incubation at 4˚C. (A) Positive control, *P. aeruginosa* ATCC® Z25.1 (B) a gelatinase-negative isolate.

(original, 2024)

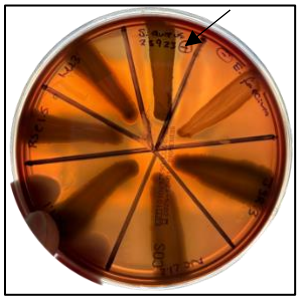


Figure 4. Hemolysins production assessed in Columbia Agar containing 5% sheep blood. Positive control, *S. aureus* ATCC® 25923 (black arrow), and non-haemolytic isolates.

(original, 2024)

For lecithinase production, isolates were inoculated in egg yolk agar composed of tryptic soy agar (VWR®, Leuven, Belgium) and 10% egg yolk emulsion (SGL, Corby, United Kingdom). Lecithinase, also known as Phospholipase C, is an enzyme that hydrolyses lecithin into phosphorylcholine and 1,2-diglyceride (McGregor et al. 1991). The determination of lecithinase activity was performed through the observation of a white, opaque halo surrounding the bacterial growth, following incubation at 37ºC for 24 hours. Negative results corresponded to the absence of a white precipitation zone, as shown in Figure 5 (Preda et al. 2021;Hwang and Park 2015). *P. aeruginosa* ATCC® 27853TM was used as the positive control and *E. coli* ATCC® 25922 as the negative control.

The casein hydrolysis test was used to determine the bacteria's ability to synthesize the proteolytic exoenzyme caseinase. Casein, a phosphoprotein, is the collective name for a family of milk proteins (Głąb and Boratyński 2017). Skim milk agar, composed of skim milk powder (VWR®, Leuven, Belgium) and bacteriological agar (VWR®, Leuven, Belgium), incubated at 37ºC for 24 hours, was used to determine the proteolytic activity of each isolate.

Positive results were considered when a transparent, clear halo was formed around the bacterial growth, indicating casein proteolysis, as shown in Figure 6. *P. aeruginosa* ATCC® 27853TM and *S. aureus* ATCC® 29213TM were used as positive and negative controls, respectively.

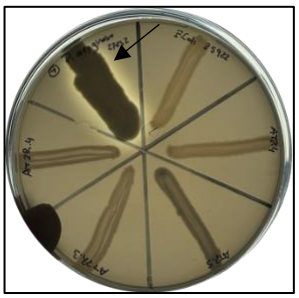


Figure 5. Lecithinase production assessed in Egg yolk agar. Positive control, *P. aeruginosa* ATCC® 27853TM (black arrow), and lecithinase-negative isolates.

(original, 2024)

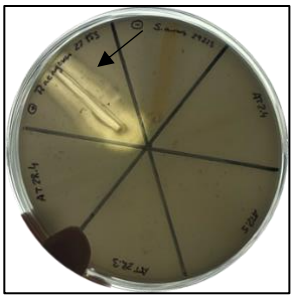


Figure 6. Proteolytic activity assessed in Skim milk agar. Positive control, *P. aeruginosa* ATCC® 27853TM (black arrow), and protease-negative isolates.

(original, 2024)

4.5 Statistical analysis

To evaluate the possible relationships between the *E. coli* isolates' antimicrobial resistance, virulence profiles and independent variables from the animals' clinical history, all the relevant data was imported and organised in a database using Microsoft® Office Excel 365 (Microsoft Corporation, Redmond, USA) and further analysed using SAS (SAS 9.4, SAS Institute Inc., Cary, NC). The independent variables tested included species, taxonomic group (Pantherinae vs Felinae), origin, sex, age, time at the sanctuary, shared enclosure, antibiotic treatment within the last 6 months, IUCN red list status and the human proximity factor (as the 4 variables detailed in Table 1). In terms of the antimicrobial resistance data analysed, solely the antibiotics enrofloxacin (ENR), gentamicin (CN), sulfamethoxazole-trimethoprim (SXT), chloramphenicol (C), ampicillin (AMP) and tetracycline (TE) were included in the analysis due to isolate 100% susceptibility to the remaining antimicrobials; amoxicillin-clavulanate (AMC), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), aztreonam (ATM) and imipenem (IPM). Regarding the data on virulence profiles, formation of biofilm was the only virulence factor detected, expressed by the isolates under study, and therefore was the only one included in the analysis.

Multivariable logistic models (PROC LOGISTIC) were used to analyse the associations between the selected independent variables mentioned above and the isolate's capability to resist antimicrobial agents. Given the ordinal nature of response, the association between the indexes calculated (Multiple Antimicrobial Resistance (MAR) and Virulence (V)) and the independent variables was analysed by fitting cumulative logistic regression models (PROC LOGISTIC). The indexes used were calculated according to the following equations: MAR Index = (Nº. antimicrobials for which an isolate tested resistant / Nº. antimicrobials tested); V. Index = (Nº. positive virulence factors / Nº. virulence factors tested) (Fernandes et al. 2024).

The Spearman correlation coefficient was used to test the relationship between each of the antimicrobials for which the isolates tested resistant, a conventional approach was then used to translate the correlation coefficients into descriptors ranging from negligible to a very strong relationship (Schober et al. 2018). This analysis was completed with PROC CORR.

A conservative approach was considered for the interpretation of the antimicrobial susceptibility data, whereby intermediate results were classified as resistant.

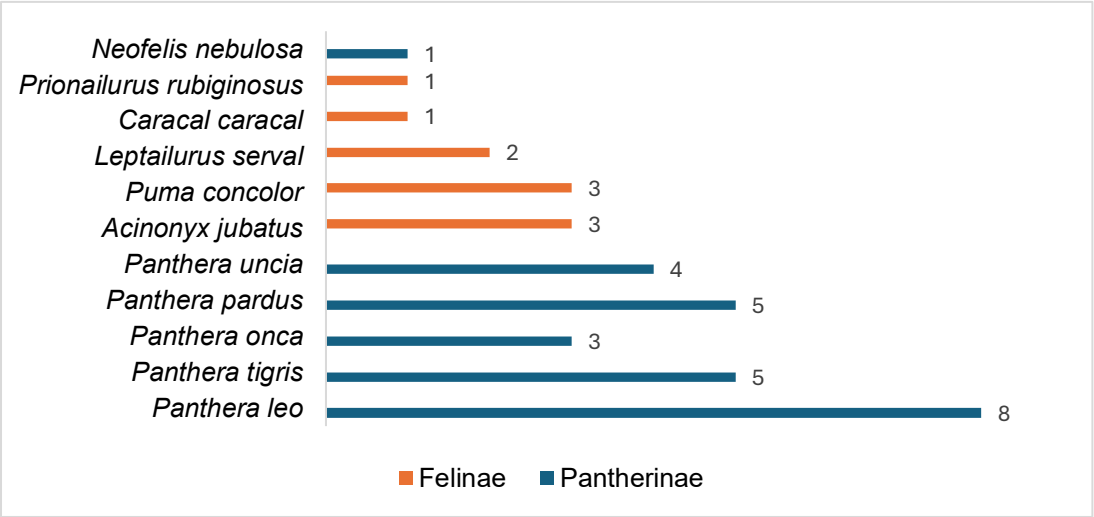
All statistical tests were conducted with a 95% confidence interval and were considered statistically significant when $p \leq 0.05$ levels of significance.

Section 5. Results

5.1 Sample characterization

In this study, a total of 36 captive non-domestic felids belonging to 11 animal species living permanently at the Big Cat Sanctuary were sampled, with lions (*Panthera leo*) representing the largest proportion of the sampled population, as shown in Graph 1. Of these, 36.1% (n = 13) were males and 63.9% (n = 23) were females. In the facility, the animals were divided into 2 groups according to their subfamily. The majority of the sampled animals were from the Pantherinae subfamily (26/36, 72.2%), commonly named 'big cats', and the remaining were from the Felinae subfamily (10/36, 27.8%), commonly named 'small cats'.

Graph 1. Species distribution in this study.



The age distribution of the sampled population ranged from 1 to 20 years old, with an average age of 10.3 years. Since age at independence and age of sexual maturity differ between individual animals as well as being species-specific, the sampled population was not categorized in accordance with these terms. The median duration of housing at the sanctuary was 7.5 years.

In terms of IUCN (International Union for Conservation of Nature) Red List categories, the animals in this study are classified as follows; 19.4% (n=7) as Critically Endangered (CR); 8.3% (n=3) as Endangered (EN); 44.4% (n=16) as Vulnerable (VU); 11.1% (n=4) as Near Threatened (NT); and 16.7% (n=6) as Least Concern (LC) (IUCN 2025).

Regarding origin, 80.6% (n = 29) of the sampled population came from zoos, 11.1% (n = 4) were born at the Big Cat Sanctuary, and 2.8% each (n = 1) were rescued from circus, private collections or rescue projects. Most animals (80.6%; n = 29) had no record of antimicrobial treatment during the six months prior to sampling; of those treated with antibiotics (19.4%; n = 7), 85.7% (n = 6) received amoxicillin, 14.3% (n = 1) marbofloxacin, and 14.3% (n = 1) doxycycline. In terms of housing, 58.3% (n = 21) were kept individually, while 41.7% (n = 15) lived in groups.

Concerning human proximity, 19.4% (n = 7) of the animals had keepers present within their enclosures while the animals were present, while 13.9% (n = 5) had direct contact with the keepers. Additionally, 50.0% (n = 18) participated in the “keeper for the day” activity, and 55.6% (n = 20) were involved in “big cat encounters,” resulting in extra opportunities for close interaction with visitors.

Table 2. List of captive felids and sample collection included in this study.

Serial	Animals		Sample Collection	
	Common name	Scientific name	Number	Type
1	African lion	*Panthera leo leo*	1	Individual
2	African lion	*Panthera leo leo*	1	Individual
3	African lion	*Panthera leo leo*	2	Composite
4	African lion	*Panthera leo leo*		
5	African lion	*Panthera leo leo*		
6	African lion	*Panthera leo leo*		
7	Asiatic lion	*Panthera leo leo*	1	Individual
8	Asiatic lion	*Panthera leo leo*	1	Individual
9	Sumatran tiger	*Panthera tigris sumatrae*	1	Individual
10	Sumatran tiger	*Panthera tigris sumatrae*	2	Individual
11	Amur tiger	*Panthera tigris altaica*	1	Individual
12	Amur tiger	*Panthera tigris altaica*	2	Individual
13	Bengal tiger	*Panthera tigris tigris*	2	Individual
14	Jaguar	*Panthera onca*	2	Individual
15	Jaguar	*Panthera onca*	2	Individual
16	Jaguar	*Panthera onca*	2	Individual
17	Amur leopard	*Panthera pardus orientalis*	1	Individual
18	Amur leopard	*Panthera pardus orientalis*	2	Individual
19	Amur leopard	*Panthera pardus orientalis*	2	Individual
20	North-Chinese leopard	*Panthera pardus orientalis*	1	Individual
21	North-Chinese leopard	*Panthera pardus orientalis*	1	Individual
22	Snow leopard	*Panthera uncia*	2	Composite
23	Snow leopard	*Panthera uncia*		
24	Snow leopard	*Panthera uncia*		
25	Snow leopard	*Panthera uncia*	1	Individual
26	Southern cheetah	*Acinonyx jubatus jubatus*	1	Individual
27	Southern cheetah	*Acinonyx jubatus jubatus*	1	Composite
28	Southern cheetah	*Acinonyx jubatus jubatus*		
29	Puma	*Puma concolor*	1	Individual
30	Puma	*Puma concolor*	1	Individual
31	Puma	*Puma concolor*	1	Individual
32	Serval	*Leptailurus serval*	1	Individual
33	Serval	*Leptailurus serval*	1	Individual
34	Caracal	*Caracal caracal*	2	Individual
35	Rusty-spotted cat	*Prionailurus rubiginosus*	1	Individual
36	Clouded leopard	*Neofelis nebulosa*	1	Individual

Legend. The scientific name category of each subspecies was based on the most recent Felidae taxonomy revision recognised by the IUCN SSC Cat Specialist Group (Kitchener et al. 2017).

5.2 Identification of Isolates

It was possible to collect a total of 41 faecal samples, 11 of which corresponded to duplicate samples. Initially, 138 bacterial isolates were obtained. Following the exclusion of morphologically similar isolates from repeated samples, 122 morphologically distinct isolates compatible with lactose fermenting *Enterobacteriaceae* in MacConkey agar were obtained and selected for further characterization.

The isolates were subjected to IMViC testing, which allowed to confirm the identification of 108 (88.5%) isolates as *E. coli*, meaning that 14 isolates did not produce results compatible with the species (Methyl red positive, Voges-Proskauer negative, Citrate negative and Indole positive) as shown in Figure 8. Four of these isolates, which were not able to produce indole, were submitted to a second IMViC testing and inoculated in Triple sugar iron (TSI), allowing to confirm that they did not correspond to *E. coli* isolates and were therefore excluded from the study. Motility, evaluated through inoculation in SIM, was exhibited by 74.1% (n = 80) of the isolates.

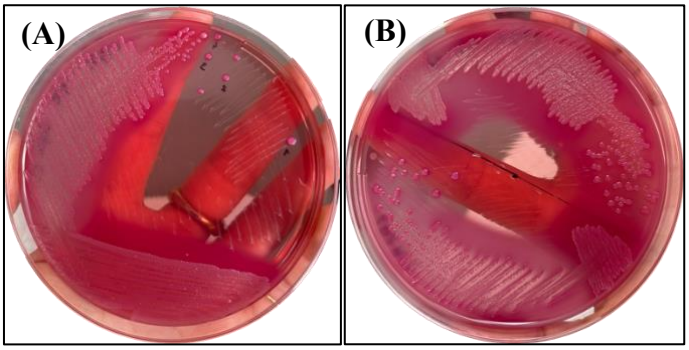


Figure 7. Results from sample inoculation isolated on MacConkey Agar Medium. (A) Initial inoculation of transport swab on MacConkey agar medium. (B) Isolation of *E. coli* suspect colonies on MacConkey agar medium.

(original, 2024)

It was possible to obtain a minimum of 1 *E. coli* isolate from every animal sampled, meaning that *E. coli* isolates were successfully obtained from all the collected samples (n = 41).

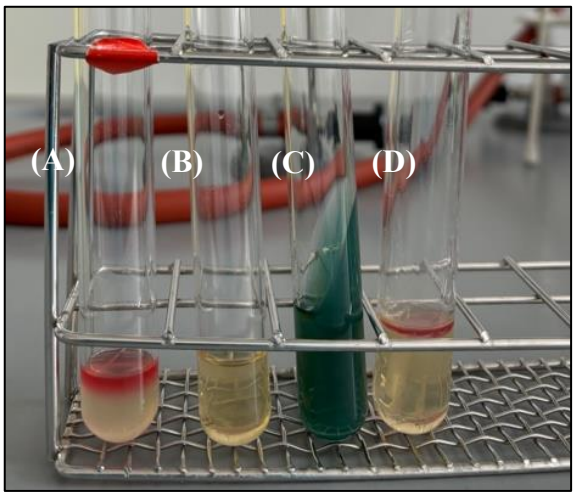


Figure 8. IMVIC series results compatible with *E. coli.* (A) Positive methyl red (MR) test post addition of methyl red reagent. (B) Negative Voges-Proskauer (VP) test post addition of reagents VP1 and VP2. (C) Negative Simmons Citrate test. (D) Positive indole test post addition of Kovac's reagent, positive motility and negative H2S production.

(original, 2024)

5.3 Antimicrobial resistance of the *Escherichia coli* isolates

In terms of the isolates' antimicrobial resistance profiles, 53.7% (n = 58) of the isolates displayed resistance or intermediate resistance to at least one of the twelve antimicrobial agents tested. The highest resistance rates were observed against tetracycline (19.4%) and ampicillin (10.2%). Among the 12 antimicrobials tested, complete susceptibility was observed for six agents across five antimicrobial classes: beta-lactam with beta-lactamase inhibitor (AMC), third-generation cephalosporins (CAZ, CTX), cephamycins (FOX), monobactams (ATM), and carbapenems (IPM).

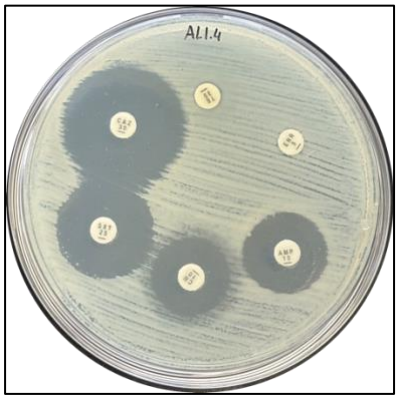


Figure 9. Kirby-Bauer disk diffusion test of an *E. coli* isolate on Mueller-Hinton agar.

(original, 2024)

In summary, 100% (n = 108) of the isolates in study showed no resistance or intermediate resistance to 50% (n = 6) of the antimicrobials tested.

All collected data on antimicrobial susceptibility testing can be found in Table 3.

Table 3. Results of the antimicrobial susceptibility test of the isolates under study to 12 antimicrobial agents.

Antimicrobial class	Antimicrobial agent	Bacterial isolates (N, %)		
		Susceptible	Intermediate	Resistant
Beta-lactam with beta-lactamase inhibitor	Amoxicillin-clavulanate (30 µg)	108 (100)	0 (0)	0 (0)
Third generation cephalosporins	Ceftazidime (30µg) / Cefotaxime (30µg)	108 (100)	0 (0)	0 (0)
Cephamycins	Cefoxitin (30µg)	108 (100)	0 (0)	0 (0)
Fluoroquinolones	Enrofloxacin (5µg)	98 (90.7)	6 (5.6)	4 (3.7)
Aminoglycosides	Gentamicin (10µg)	73 (67.6)	35 (32.4)	0 (0)
Monobactams	Aztreonam (30µg)	108 (100)	0 (0)	0 (0)
Folate pathway inhibitors	Sulfamethoxazole-trimethoprim (25µg)	101 (93.5)	0 (0)	7 (6.5)
Phenicols	Chloramphenicol (30µg)	107 (99.1)	0 (0)	1 (0.9)
Penicillins	Ampicillin (10µg)	90 (83.3)	7 (6.5)	11 (10.2)
Tetracyclines	Tetracycline (30µg)	87 (80.6)	0 (0)	21 (19.4)
Carbapenems	Imipenem (10µg)	108 (100)	0 (0)	0 (0)

Legend. Number of isolates (N), Percentage (%).

In this study, 10 isolates (9.3%) were classified as MDR due to acquired non-susceptibility to at least one antimicrobial in 3 or more antimicrobial categories. Among the MDR *E. coli* isolates, tetracycline and ampicillin resistance was the highest (10/10, 100%), followed by sulfamethoxazole-trimethoprim and gentamicin (5/10, 50%) and chloramphenicol (1/10, 10%). In terms of the animal species, Jaguars (*Panthera onca*; 2/3, 66.7%) and Servals (*Leptailurus serval*; 2/2, 100%) presented the highest percentage of MDR isolates. Among the 10 MDR *E. coli* isolates, 7 were obtained from samples of 4 animal species from the Pantherinae subfamily and 3 from 2 species from the Felinae subfamily. Four out of ten (40%) of these isolates were recovered from host species classified as Vulnerable (VU) on the IUCN Red List.

The mean value of the MAR index was 0.07 (SD = 0.08), ranging from 0.00-0.33. No significant differences were observed between the MAR index values or the MDR profile of the *E. coli* isolates and any of the 13 independent variables tested.

The association between the isolates' AMR profiles and the 13 independent variables: species, taxonomic group (Pantherinae vs Felinae), origin, sex, age, time at the sanctuary, shared enclosure, antibiotic treatment within the last 6 months, IUCN red list status, presence of keepers in enclosure, direct contact, involvement of the animals in 'keeper for the day' and 'big cat encounter' activities, was also evaluated. It was possible to observe a statistically significant association between isolates resistance to enrofloxacin (ENR) and the sex of the sampled animals ($p = 0.015$) and, according to the odds ratio, females were shown to have a lower probability of carrying *E. coli* resistant to enrofloxacin (OR = 0.167). Furthermore, a significant association between *E. coli* isolates resistance to gentamicin (CN) and the variable 'keeper in enclosure with animal present' ($p = 0.0008$) was observed. According to the odds ratio, it is possible to conclude that animals present in the enclosure at the same time as keepers have a higher probability of carrying *E. coli* isolates resistant to gentamicin (OR = 0.103). Isolate resistance to ampicillin (AMP) showed a statistically significant association with the variable 'shared enclosure' ($p = 0.0446$), suggesting animals housed individually had a lower probability of carrying an *E. coli* isolate resistant to AMP (OR = 0.282). Additionally, a p value of 0.0608 was obtained when analysing the relationship between *E. coli* resistance to tetracycline and the variable 'keeper for the day'. This value, slightly above the statistically significant threshold ($p \leq 0.05$), suggests a marginally significant or tendency towards association between these two variables and therefore no definitive conclusion can be drawn regarding this association.

By analysing the possible relationship between resistance to different antibiotics within the same bacterial isolate, a moderately positive correlation was found between tetracycline and ampicillin resistance (0.490). According to the conventional interpretation of correlation coefficients proposed by Schober et al. (2018), a moderate correlation is defined as a value between 0.40-0.69. Other moderately positive correlations were identified between ampicillin and sulfamethoxazole-trimethoprim resistance (0.488), as well as between tetracycline and sulfamethoxazole-trimethoprim resistance (0.455). The remaining correlations found between resistance to other antimicrobial agents were deemed too weak, below the cut-off value of 0.39, and therefore not significant.

5.4 Virulence profiles of the isolated *Escherichia coli*

Regarding the isolates' phenotypic virulence profiles, only 8.3% (n = 9) of the isolates in study were able to phenotypically express one of the virulence factors tested, namely biofilm formation. Hence, 91.7% (n = 99) of the *E. coli* isolates under study were found not able of

phenotypically expressing the remaining virulence-associated enzymes tested: protease, DNase, gelatinase, lecithinase and hemolysins.

Regarding the origin of the 9 biofilm-producing isolates, Jaguars (*Panthera onca*; 2/3, 66.7%) presented the highest frequency of positive isolates, and the Caracal (*Caracal caracal*) had the highest number of positive isolates detected per individual (n = 2).

The mean value of the V index was 0.01 (SD = 0.05), ranging from 0.00-0.17. No statistically significant associations were detected between any of the independent variables tested and biofilm production or the Virulence index ($p > 0.05$). Moreover, no statistically significant correlations were found between the MAR index and the Virulence index ($p > 0.05$).

Section 6. Discussion

6.1 Interpretation of Findings

The emergence of antimicrobial resistance is a growing threat to public health worldwide that spans across both human and veterinary medicine (Laborda et al. 2022; Hickson et al. 2025). Although wildlife has long been recognized as a reservoir of resistant pathogens, only in recent years has research begun to focus more intensively on their role in AMR dynamics, with particular attention to the influence of animals' captivity on resistance profiles (Huang 2022; Cai et al. 2024).

Among bacterial pathogens, and beyond acquired resistance, Gram-negative species have been described as exhibiting intrinsic resistant to several antibiotics, to a greater extent when compared to Gram-positive species, making multidrug-resistant Gram-negative bacteria especially concerning for modern medicine practises (Kulasooriya et al. 2016; Hickson et al. 2025).

The presence of ARB in captive wildlife represents a risk from multiple perspectives, including interspecies dissemination within zoological institutions and transmission across geographical locations associated with species conservation projects. While free-living wildlife is generally considered to play a limited role in the emergence of AMR due to minimal exposure to selective pressures derived from antibiotics and biocides, this role changes markedly under captive conditions (Laborda et al. 2022). In wild animal care facilities, wildlife are exposed to increased anthropogenic pressures, due to close proximity and occasional direct contact with humans, as well as increased exposure to antimicrobial treatments and environmental stressors, all of which may contribute to the persistence, transmission, and even emergence of resistant bacteria (Wales and Davies 2015). Evidence shows that wildlife housed in captivity

may carry a higher prevalence of ARB containing clinically relevant ARGs, a pattern amplified by human activity and husbandry practises (Tsukayama et al. 2018). Consequently, the local dissemination of AMR within sanctuaries is of particular concern, underscoring its importance within a One Health perspective. However, despite the evident public health implications, targeted surveillance of AMR in managed wildlife populations remains limited (Feng et al. 2023).

This study was conducted to evaluate and provide insight on the prevalence of antibiotic resistance and virulence factors of *E. coli* from captive sanctuary-housed non-domestic felids. The findings revealed variable resistance profiles across a range of felid species and suggested how they may be influenced by a series of selected independent variables within captive settings. To the best of the authors' knowledge, this is the only study to report the occurrence of antimicrobial resistance exclusively in captive non-domestic felids within UK wildlife sanctuaries.

The resistance patterns observed in this study, particularly the predominance of resistance to broad-spectrum antibiotics such as tetracycline and ampicillin, reflect previously reported trends among wild felids. Similar findings reporting high incidence rates in these mentioned antibiotics have been documented, such as the studies by Ghosh et al. (2019), Xue et al. (2013) and Li, Lan, et al. (2024), focusing on *E. coli* isolates from captive Bengal tigers in Bangladesh, captive Amur tigers in China, as well as in free-ranging Amur tigers and North Chinese leopards, respectively. In contrast to these previous studies on captive and free-ranging wild felids, where resistance to chloramphenicol, sulfamethoxazole–trimethoprim and imipenem is reported, isolates in the present study remained largely susceptible to these critically important antimicrobials. These differences may reflect a range of contributing factors, which will be further explored in the following discussion.

Although tetracycline has been reported as a widely used antimicrobial in veterinary medicine, as well as in zoo settings (Kim et al. 2024; Schmartz et al. 2024), it was not documented amongst the most commonly used antimicrobials at the place of sampling for this study. According to the local medical records obtained through the Zoological Information Management System (ZIMS), only 7 of the 36 felids sampled received antibiotic treatment in the 6 months preceding sample collection. Also, the association between recent (6 months) antibiotic treatment and the prevalence of resistant isolates was not statistically significant. This may reflect the small sample size, the relatively short time frame considered and the frequency of treatments, besides other factors beyond the scope of this study, such as prior antimicrobial use outside of the sanctuary due to inaccessibility of clinical records preceding the animals' arrival at the place of sampling. Collectively, these observations support the hypothesis that factors other than local antimicrobial treatment practises may have contributed to the resistance profiles observed.

Furthermore, the consistent high prevalence of tetracycline resistance reported in wildlife AMR studies has been described to be potentially linked to its extensive use in livestock production. This theory is supported by studies reporting a high diversity of tetracycline resistant genes in livestock waste, which may act as a facilitating vehicle for their emergence and spread in the environment (Pazra et al. 2023). The prevalence of tetracycline resistance is also frequently associated with the anthropization of landscapes, reflecting the impact of human activity on the predominance of tetracycline-specific ARGs (Sacristán et al. 2020). Additionally, the carnivorous diet of wild felids may also be an important transmission route (Feng et al. 2023), although, to the best of the author's knowledge, currently there is no literature available directly linking contamination rates of resistant *E. coli* with the dietary regimes of captive wildlife, underscoring the importance of future research in this area.

With respect to the present study, if the principal source of resistant bacteria was the contaminated food, it would be expected that the isolates originating from animals of the same species would exhibit similar resistance profiles. The absence of such similarities suggests that foodborne transmission alone is unlikely to account for the observed patterns, indicating the influence of an additional or a mix of factors in the resistance profiles of the isolates under study. In addition, the contribution of vectors such as insects and rodents cannot be overlooked, representing a possibility which is especially relevant in the rural context of the sanctuary (Yin et al. 2022; Devanathan et al. 2024). However, given that the sampling focused on wild felids from a range of geographical locations and originated from diverse wildlife care facilities, each with distinct treatment and management protocols and diverse levels of human–animal interaction, determining the exact sources of ARB they harbour would be challenging.

Another factor to consider is the widespread use of biocides in animal care environments. Disinfectants containing quaternary ammonium compounds (QAC), commonly used in animal hospitals and zoos, as well as in the animal sanctuary from this study, are routinely used for the disinfection of food preparation surfaces, animal enclosures and foot dips. While these practises represent an important biosecurity measure aimed at reducing antimicrobial use, QACs have also been described as 'agents with the potential for co-selection of antimicrobial resistance' (Wales and Davies 2015). QAC-resistance determinants are frequently located on mobile genetic elements such as plasmids, which often carry additional ARGs linked to a decreased susceptibility of bacterial isolates to antibiotics (Li, Brejnrod, et al. 2024). Furthermore, exposure to QACs at subinhibitory concentrations has been shown to promote HGT (Liu et al. 2023) and create conditions which enhance mutagenesis (Kim et al. 2018), both of which may contribute to an increased prevalence of MDR bacterial isolates. Although in veterinary medicine the majority of research on this topic has focused primarily on the livestock-food chain industry (Wales and Davies 2015), it is plausible that similar mechanisms occur in captive wildlife settings. While this hypothesis lies beyond the scope of

the present study, published evidence suggests that biocide use could be considered a potential contributor to the resistance profiles observed here (Kim et al. 2018).

Reports of *E. coli* resistance to three or more antimicrobials from different antimicrobial categories in studies focusing solely on wild felid species remain notably scarce across available literature. A recent study conducted in India documented a 21% prevalence of MDR *E. coli* across animals from four species within the Pantherinae subfamily (Shukla et al. 2022), a markedly higher prevalence in comparison with the results from this present study (9.3%). Evident disparities in MDR bacterial prevalence have also been described in AMR studies focusing on wildlife rehabilitation centres, reporting resistance rates as high as 90% in Chile (Baros Jorquera et al. 2021) and 71% in Spain (Vidal et al. 2017). Also, a study performed in a zoo in Belgium has reported an MDR prevalence in *E. coli* of 64% (De Witte et al. 2021). In contrast, the isolation of a high prevalence of resistant *E. coli* strains from carnivores has been consistently reported in the literature, representing a widely recognised trend (Jobbins and Alexander 2015; Bamunusinghage et al. 2022; Kim, Kim, et al. 2024).

All the MDR isolates obtained in this study were resistant to tetracycline and ampicillin, while half of them were resistant to sulfamethoxazole-trimethoprim and gentamicin. These findings align with previous reports in captive wildlife (De Witte et al. 2021; Medina et al. 2024). The origins of this resistance pattern observed remain unclear, given only 30% (3/10) of the resistant isolates were obtained from animals with a recent history of antibiotic treatment (within the six months prior to sampling). This suggests that the emergence of MDR in this sanctuary is likely multifactorial, once again extending beyond antibiotic treatment practises. Notably, moderately positive statistical correlations were observed between resistance to tetracycline, ampicillin and sulfamethoxazole-trimethoprim. Such associations may be consistent with the co-location of ARGs on the same mobile genetic element, potentially leading to co-selection (Ozawa et al. 2023). The presence of ARGs associated with resistance to these antibiotics has been documented through molecular testing and associated with phenotypic resistance regarding ampicillin and tetracycline (Chin et al. 2024). Furthermore, selective pressure from common stressors, including antimicrobial and biocide exposure, may promote the persistence and propagation of mobile genetic elements harbouring multiple ARGs, thereby providing a plausible explanation for the observed co-occurrence of resistance (Gullberg et al. 2014).

In this study, 70% of the MDR *E. coli* isolates were obtained from animals within the Pantherinae subfamily, this observation may indicate a possible association between taxonomic group and sanctuary management practises. One possible explanation for this suggestion is the participation of these animals in the ‘animal activities’ offered by the sanctuary, in contrast to the Felinae species (with the exception of cheetahs). With the objective of enhancing public education and engagement, the increased anthropogenic activity

associated with these activities may contribute to the observed association; however, it is important to refer that no statistically significant difference in MDR *E. coli* prevalence was found between the results from the two subfamilies. This further supports the idea that the resistance profiles observed in this study are unlikely to be explained by a single factor, particularly as two taxonomically distinct species, jaguars and servals, exhibited the highest percentage of MDR isolates.

Furthermore, four of the ten MDR isolates obtained in this study originated from animals classified as 'Vulnerable' under the IUCN Red List. While this may partly reflect the species present in the sanctuary, given that 44.4% of the animals analysed hold this status, it also raises concerns regarding additional health challenges faced by threatened taxa, associated with antimicrobial resistance. Such findings emphasize the importance of integrating AMR monitoring into wildlife conservation practises (Benavides et al. 2024; Vezeau and Kahn 2024), as resistant infections and the diminishing availability of effective antimicrobial treatments compromise both animal welfare and the success of *ex-situ* conservation programs (Ramey and Ahlstrom 2023).

Interestingly, in this study, females statistically exhibited a lower probability of carrying *E. coli* resistant to enrofloxacin. Although this finding may also be attributed to the small sample size and be biased by the uneven demographic distribution, sex-related differences cannot be excluded as having a potential role in AMR dynamics. However, evidence for sex as a determinant of AMR patterns in wildlife studies remains very limited and is frequently not considered to be a significant predictor (Höcketstaller et al. 2025). Existing literature focusing on AMR in wildlife more commonly identify factors such as 'social degree' and 'age' as stronger predictors of antimicrobial-resistant *E. coli* (Miller et al. 2019), as well as proximity to anthropogenetic activities (Akwongo et al. 2025). Similar conclusions have also been reported in human medicine, acknowledging gender as a small contributor to resistome composition (Salehi et al. 2025).

Another noteworthy finding from this study was the significant association found between *E. coli* isolates resistant to gentamicin and the independent variable 'keeper in enclosure with animal present'. At this animal sanctuary, animal keepers routinely enter enclosures while certain animal species remained inside, rather than safely moving the animals beforehand, which is a standard practise in the management of captive non-domestic felids. This approach is exclusively adopted with species belonging to the Felinae subfamily, for example cheetahs and servals. Gentamicin, an aminoglycoside widely used in human medicine for treating severe gram-negative bacterial infections, has been previously associated with increased anthropogenic exposure in wildlife (Zeballos-Gross et al. 2021) and also in domestic animals, highlighting the association between human-pet households and aminoglycoside ARG abundance (Yang et al. 2023). This association may be mediated by

direct transfer of resistant bacteria from keepers to animals, contamination of enclosure surfaces or shared equipment, or the horizontal transfer of human-associated resistance genes into the animals' microbiomes, potentially influencing the dissemination of gentamicin resistance. While this finding should be interpreted with caution due to the study's exploratory nature, small sample size and absence of genomic techniques to explore transmission dynamics in this setting, this association supports the well-established concept that anthropogenic pressures within captive settings can influence resistance profiles in wildlife species.

Another probable explanation for the acquisition of resistance among bacteria isolated from captive wild animals is the occurrence of horizontal transmission between bacteria from different animal groups, a process influenced by the housing structures adopted in zoos and wildlife sanctuaries. In this study, a statistically significant association was observed between *E. coli* isolates resistant to ampicillin and animals housed in shared enclosures. This finding indicates that zoo housing practises may facilitate the horizontal propagation of ARB, thereby shaping resistance profiles in captive populations. Similar associations have been reported in previous studies in which ARB across zoo animals has been linked to housing and husbandry factors as a potential transmission route (Sealey et al. 2023; M. Kim et al. 2024). These results reinforce the view that zoo practises can create conditions conducive to interspecies dissemination of ARB.

Regarding the phenotypic virulence profiles observed, the results were unexpectedly low, with only 8.3% of the isolates expressing biofilm formation. For wild felids specifically, targeted studies on the pathogenic potential of *E. coli* are limited. A case study characterizing pathogenic *E. coli* strains of two leopard species revealed a wide range of virulence factors and raised concerns about the co-occurrence of MDR and virulent strains in captivity (Carvalho et al. 2012). Similarly, a study on free-ranging large felids reported a high prevalence of virulence genes per sample, such as those associated with the production of Shiga toxin, and identified isolates of *E. coli* serotypes associated with urinary tract infections and enteric disease. These findings suggest a high pathogenetic potential among isolates, although the contribution to disease risk and species decline in wild populations was left unclear (Li et al. 2024). In contrast, the low prevalence of phenotypic virulence factors observed in the present study may reflect methodological limitations, as culture-based techniques primarily capture expressed virulence traits and may underestimate underlying pathogenic potential when compared to molecular approaches. Nonetheless, the limited detection of virulence expression by the *E. coli* isolates from the studied animals may indicate a low burden of pathogenic *E. coli*, which could be viewed as encouraging from an animal health and welfare perspective in this sanctuary. Supporting this interpretation, the MAR Index and Virulence Index were not significantly correlated, suggesting that isolates with higher levels of antimicrobial resistance,

did not generally possess a greater number of virulence factors. While this may imply that increased AMR burden does not necessarily exacerbate pathogenic potential of bacteria present in captive wild felids, an encouraging finding from a conservation medicine perspective, alternative associations beyond the detection limits of this study cannot be excluded.

6.2 Study Limitations

Regarding the limitations of this study, improvements could have been made regarding the study design, in particular in terms of sampling constraints.

The cross-sectional design of this study, with samples collected at only three points in time, limited the ability to capture temporal variation or establish a sequence of events. It was therefore not possible to determine whether animals, other than those born in the sanctuary, carried resistant bacteria prior to arrival or acquired them at the sanctuary.

Furthermore, opportunistic non-invasive faecal sampling also introduced constraints, as “sample freshness” (time since defecation) could not always be guaranteed. This may have affected bacterial viability and growth, potentially underestimating resistance prevalence. Another limitation related to the nature of the faecal samples is the sampling strategy, as faecal material was collected from the animal enclosures rather than directly from the animals. Although the samples were obtained from the interior of the faecal material collected in order to avoid environmental contamination, this possibility cannot be completely excluded, as resistant *E. coli* may also be originated from the soil, water or the enclosure surfaces. Consequently, this makes it difficult to attribute the observed resistance profiles to a single origin.

Additionally, the sample size included in this study was relatively low (n = 36), with an uneven representation across animal species. This limited a more robust statistical analysis and may partially explain the discrepancies between the present findings and those from previously published literature. Moreover, differences in zoo management practices, enclosure design, and husbandry routines further restrict the extrapolation of these results and reproducibility beyond this single sanctuary.

Another limitation of this study includes the absence of molecular analysis beyond the culture-based methods and antimicrobial susceptibility testing outlined in the Materials and Methods. The incorporation of molecular techniques, such as PCR and sequencing, would have enabled the characterisation of underlying resistance mechanisms and the identification of potential associations between human-derived bacteria and the animal isolates analysed, thereby providing additional information on possible transmission pathways.

Furthermore, the reliance on culture-based methods to identify *E. coli* isolates presents some challenges. These techniques are limited in their sensitivity due to the wide strain diversity present in both the animal samples and the environment, often leading to the overrepresentation of the most abundant or easily cultivable strains while overlooking less prevalent or fastidious variants. In addition, culture-based methods alone are unable to differentiate between commensal and pathogenic *E. coli* strains, which limits the ability to interpret the clinical significance of the obtained isolates.

Taken together, these limitations require a cautious interpretation of the resistance profiles and virulence factors observed in this study.

6.3 Identification of Knowledge Gaps and Areas for Future Research

Despite the growing focus on antimicrobial resistance in wildlife, there is still a critical need to increase knowledge on the influence of antibiotic exposure and anthropogenic impact on wild animals living in captivity. Zoos, animal sanctuaries and wildlife rehabilitation centres represent unique settings where human activity and animal care intersect, yet the extent to which these controlled environments shape the resistome of wild species is still poorly understood.

Collaboration among wild animal care centres across diverse geographical regions and wildlife management contexts would enable comparative research, allowing for a more comprehensive understanding of the mechanisms driving resistance acquisition and dissemination, as well as the potential for interspecies and human-animal transmission within *ex-situ* conservation organisations.

Future research focusing on wildlife in captivity should extend beyond the evaluation of direct antibiotic exposure as the primary driver of resistance and explore the influence of additional anthropogenic factors. Comparative multi-factor study designs focusing on host- and captivity-associated independent variables, such as the one employed in this study, provide a useful framework for assessing how local management practises shape exposure pathways, thereby allowing the development of more targeted prevention strategies. Particular attention should be given to the role of raw meat-based diets in the introduction of resistant bacteria into captive carnivore populations, as well as to the potential transmission routes of AMR strains between wildlife and sanctuary personnel, predominantly in those in which close contact is a frequent practise. Incorporating the assessment of the influence of such independent factors in wildlife AMR studies would provide valuable insights for both animal welfare and potential occupational and public health risks.

Addressing these focus areas through routine surveillance and context-specific investigations will be essential for advancing our understanding of AMR dynamics in captive wildlife and guiding effective mitigation strategies.

Section 7. Conclusion

This study characterised the antimicrobial resistance (AMR) and phenotypic virulence profiles of *Escherichia coli* isolated from captive non-domestic felids. Overall, resistance levels were low to moderate, with only a small proportion of multidrug-resistant isolates detected, and virulence traits were limited to biofilm formation. Despite this, the presence of resistant strains underscores the role of captive wild felids as potential reservoirs of AMR and their contribution to resistance dynamics within human-managed environments.

Associations between resistance patterns and captivity-related factors, including human proximity and enclosure type, suggest that management practises and environmental conditions within these settings may influence the resistome of these populations. While these findings are preliminary, they highlight important hypotheses for future investigation and emphasise the need for broader, comparative, multi-institutional studies.

Since the use of antibiotics is an inherent component of veterinary care, with zoo and wildlife medicine not being an exception, understanding their impact on microbial communities, as well as other anthropogenic pressures present in captive environments, offers valuable context to ensure animal care practises align with both health and welfare priorities.

Routine pathogen monitoring, with antimicrobial resistance testing at the forefront, must be recognised as a core component of conservation management strategies, as it enables the early detection of emerging threats and informs evidence-based decision-making.

The implementation of an integrated approach to AMR mitigation in captive settings, encompassing antimicrobial stewardship, hygiene and sanitation, and broader management and husbandry practises, is relevant across all managed wildlife populations. However, the findings of this study highlight the particular importance of such measures in wild felids, given the consistently reported resistance profiles in carnivores and the likelihood of more frequent exposure to anthropogenic pressures associated with their comparatively long lifespans in captivity. Importantly, such measures should aim to minimise anthropogenic selective pressures and mitigate cross-species transmission risks, thereby reinforcing the principles of One Health.

Section 8. Annexes

Annexe 1 – Metadata of sampled animals (n=36)

Table 4. Descriptive data of the sampled animals included in this study (n = 36).

Sample ID	Animal ID	Sampling Type	Scientific Name	Time at Sanctuary [1]	Sex	Age	Antimicrobial treatment [2]	IUCN Status	Origin	Shared enclosure	Human Proximity Factor			
											Keeper in enclosure	Direct contact	"Keeper for the day" [3]	"Big cat encounter" [3]
AL1	AL1	Individual	*Panthera leo leo*	8	M	14	-	Vulnerable	Circus	N	N	N	N	Y
AL2	AL2	Individual	*Panthera leo leo*	1.5	M	8	Doxycycline + Amoxicillin	Vulnerable	Zoo	Y	N	N	Y	Y
AL3	AL3	Individual	*Panthera leo leo*	1.5	F	8	-	Vulnerable	Zoo	Y	N	N	Y	Y
AL4	AL4	Individual	*Panthera leo leo*	0.083	F	3	-	Vulnerable	Rescue Project	N	N	N	N	N
WL1	WL1_1	*Composite*	*Panthera leo leo*	11	F	11	-	Vulnerable	Zoo	Y	N	N	N	Y
WL1	WL1_2	*Composite*	*Panthera leo leo*	11	F	11	-	Vulnerable	Zoo	Y	N	N	N	Y
WL1	WL1_3	*Composite*	*Panthera leo leo*	11	F	11	-	Vulnerable	Zoo	Y	N	N	N	Y
WL1	WL1_4	*Composite*	*Panthera leo leo*	11	F	11	-	Vulnerable	Zoo	Y	N	N	N	Y
ST1	ST1	Individual	*Panthera tigris sumatrae*	2.75	F	18	-	Critically endangered	Zoo	N	N	N	Y	Y
ST2	ST2	Individual	*Panthera tigris sumatrae*	19	F	20	Amoxicillin	Critically endangered	Zoo	N	N	N	N	Y
AT1	AT1	Individual	*Panthera tigris tigris*	6	M	13	Amoxicillin	Endangered	Zoo	N	N	N	N	Y

AT2	AT2	Individual	*Panthera tigris altaica*	12	F	12	-	Endangered	BCS	N	N	N	N	Y
AT3	AT3	Individual	*Panthera tigris altaica*	1.083	M	2	-	Endangered	Zoo	N	N	N	Y	Y
J1	J1	Individual	*Panthera onca*	5	F	7	-	Near threatened	Zoo	Y	N	N	Y	Y
J2	J2	Individual	*Panthera onca*	6	M	7	-	Near threatened	Zoo	Y	N	N	Y	Y
J3	J3	Individual	*Panthera onca*	7	F	7	Marbofloxacin	Near threatened	Zoo	N	N	N	Y	Y
LP1	LP1	Individual	*Panthera pardus orientalis*	4	M	4	-	Critically endangered	Zoo	N	N	N	Y	Y
LP2	LP2	Individual	*Panthera pardus orientalis*	17	F	19	-	Critically endangered	Zoo	N	N	N	Y	N
LP3	LP3	Individual	*Panthera pardus orientalis*	2	F	6	-	Critically endangered	Zoo	N	N	N	Y	Y
NCL1	NCL1	Individual	*Panthera pardus orientalis*	16	M	18	Amoxicillin	Critically endangered	Zoo	N	N	N	Y	N
NCL2	NCL2	Individual	*Panthera pardus orientalis*	16	F	17	Amoxicillin	Critically endangered	Zoo	N	N	N	Y	N
SL1	SL1_1	*Composite*	*Panthera uncia*	13	F	14	-	Vulnerable	Zoo	Y	N	Y	N	N
SL1	SL1_2	*Composite*	*Panthera uncia*	1	F	1	-	Vulnerable	BCS	Y	N	Y	N	N
SL1	SL1_3	*Composite*	*Panthera uncia*	1	M	1	-	Vulnerable	BCS	Y	N	Y	N	N
SL2	SL2	Individual	*Panthera uncia*	11	M	13	-	Vulnerable	Zoo	N	N	N	N	N
CT1	CT1	Individual	*Acinonyx jubatus jubatus*	7	F	7	-	Vulnerable	Zoo	N	Y	Y	Y	Y

CT2	CT2_1	*Composite*	*Acinonyx jubatus jubatus*	1	M	6	Amoxicillin	Vulnerable	Zoo	Y	Y	Y	Y	Y
CT2	CT2_2	*Composite*	*Acinonyx jubatus jubatus*	1	M	6	-	Vulnerable	Zoo	Y	Y	Y	Y	Y
P1	P1	Individual	*Puma concolor*	16	F	17	-	Least concern	Zoo	Y	N	N	Y	N
P2	P2	Individual	*Puma concolor*	16	F	17	-	Least concern	Zoo	Y	N	N	Y	N
P3	P3	Individual	*Puma concolor*	12	F	13	-	Least concern	Private collection	N	N	N	Y	N
S1	S1	Individual	*Leptailurus serval*	13	F	14	-	Least concern	Zoo	N	Y	N	N	N
S2	S2	Individual	*Leptailurus serval*	12	F	12	-	Least concern	BCS	N	Y	N	N	N
C1	C1	Individual	*Caracal caracal*	10	M	11	-	Least concern	Zoo	N	N	N	N	N
RSC1	RSC1	Individual	*Prionailurus rubiginosus*	0.67	M	6	-	Near threatened	Zoo	N	Y	N	N	N
CL1	CL1	Individual	*Neofelis nebulosa*	0.3	F	7	-	Vulnerable	Zoo	N	Y	N	N	N

Legend. [1] Data expressed in years; [2] Antibiotic treatment within the 6 months prior to sampling; [3] Public-interactive activities offered by the sanctuary; F: female; M: male; BCS: Big Cat Sanctuary; N: no; Y: yes.

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