Science of the Total Environment 952 (2024) 175866

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Zoonotic potential of urban wildlife faeces, assessed through metabarcoding

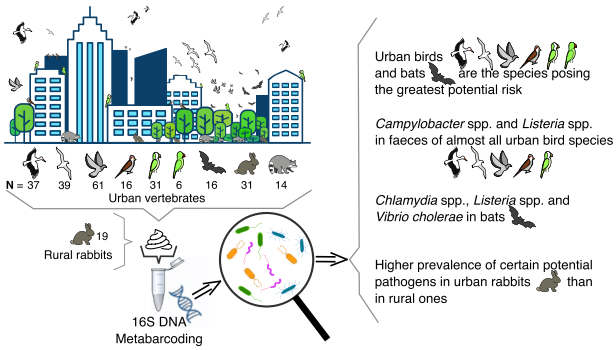
Xabier Cabodevilla [a,b,c], Juan E. Malo [a,e], Daniel Aguirre de Cárcer [d], Julia Zurdo [a,e], Rubén Chaboy-Cansado [d], Alberto Rastrojo [d], Francisco J. García [f], Juan Traba [a,e,*]

[a] Terrestrial Ecology Group (TEG-UAM), Department of Ecology, Universidad Autónoma de Madrid, Madrid, Spain
[b] Conservation Biology Group, Landscape Dynamics and Biodiversity Program, Forest Science and Technology Centre of Catalonia (CTFC), Solsona, Spain
[c] Department of Zoology and Animal Cell Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Alava, Spain
[d] Microbial and Environmental Genomics Group, Department of Biology, Universidad Autónoma de Madrid, Madrid, Spain
[e] Centro de Investigación en Biodiversidad y Cambio Global, Universidad Autónoma de Madrid (CIBC-UAM), Madrid, Spain
[f] Biodiversity Monitoring Group, Department of Biodiversity, Ecology and Evolution, Complutense University of Madrid, Jose Antonio Novais, 12, Madrid, 28040, Spain

HIGHLIGHTS

- Among urban species, studied birds' and bats' faeces pose a higher potential zoonotic risk.
- *Campylobacter* spp. and *Listeria* spp. in faeces of nearly all urban bird species
- *Chlamydia* spp., *Listeria* spp. and *Vibrio cholerae* in bats
- Higher prevalence of certain potential pathogens in urban rabbits than in rural ones
- Metabarcoding can be a valuable tool for screening potentially zoonotic organisms.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Kyle Bibby

Keywords:
Campylobacteriosis
Listeriosis
Monitoring systems
Pigeon
House sparrow
Zoonotic pathogens

ABSTRACT

Monitoring zoonoses in urban environments is of great relevance, where the incidence of certain pathogens may be higher and where population density makes the spread of any contagious disease more likely. In this study we applied a metabarcoding approach to study potentially zoonotic pathogens in faecal samples of 9 urban vertebrate species. We applied this methodology with two objectives. Firstly, to obtain information on potential pathogens present in the urban fauna of a large European city (Madrid, Spain) and to determine which are their main reservoirs. In addition, we tested for differences in the prevalence of these potential pathogens between urban and rural European rabbits, used as ubiquitous species. Additionally, based on the results obtained, we evaluated the effectiveness of metabarcoding as a tool for monitoring potential pathogen. Our results revealed the presence of potentially zoonotic bacterial genera in all studied host species, 10 of these genera with zoonotic species of mandatory monitoring in the European Union. Based on these results, urban birds (especially house sparrows and pigeons) and bats are the species posing the greatest potential risk, with *Campylobacter* and *Listeria*

* Corresponding author at: Terrestrial Ecology Group (TEG-UAM), Department of Ecology, Universidad Autónoma de Madrid, Madrid, Spain.
E-mail address: juan.traba@uam.es (J. Traba).

https://doi.org/10.1016/j.scitotenv.2024.175866
Received 30 May 2024; Received in revised form 27 August 2024; Accepted 27 August 2024
Available online 31 August 2024

genera in birds and of *Chlamydia* and *Vibrio cholerae* in bats as most relevant pathogens. This information highlights the risk associated with fresh faeces from urban wildlife. In addition, we detected *Campylobacter* in >50 % of the urban rabbit samples, while we only detected it in 11 % of the rural rabbit samples. We found that urban rabbits have a higher prevalence of some pathogens relative to rural rabbits, which could indicate increased risk of pathogen transmission to humans. Finally, our results showed that metabarcoding can be an useful tool to quickly obtain a first screening of potentially zoonotic organisms, necessary information to target the monitoring efforts on the most relevant pathogens and host species.

1. Introduction

Emerging infectious diseases, especially those with zoonotic potential, are a growing threat to human health (Jones et al., 2008; Morse et al., 2012; Rohr et al., 2019). Zoonoses are infectious diseases that are transmitted from nonhuman animals to humans (Palmer et al., 1998; Taylor et al., 2001). These diseases can be caused by viruses, bacteria or eukaryotic parasites (fungi, protozoa or helminths) and are transmitted directly, through ingestion of contaminated food or water, or through vectors such as mosquitoes or ticks (Taylor et al., 2001; Jones et al., 2008; Rahman et al., 2020). It is estimated that more than half of known human pathogens may be zoonotic (Taylor et al., 2001; Woolhouse and Gowtage-Sequeria, 2005; Mackenstedt et al., 2015). Many zoonoses have their origin in wildlife due to dynamic interactions between human populations, wildlife and livestock. As human populations advance in their occupation of hitherto virgin habitats, interactions at the animal-human interface increase, facilitating the emergence and spread of zoonoses worldwide (Magouras et al., 2020). Besides, factors such as globalisation, agricultural intensification, climate change and underfunded health care systems seem to favour the emergence of zoonotic epidemics (Walsh et al., 2020).

For these reasons, zoonoses have gained global prominence over the last decades, highlighting the need to establish preventive monitoring mechanisms (Carlson et al., 2021). In the European Union, Directive 2003/99/EC of the European Parliament on the monitoring of zoonoses and zoonotic agents stipulates which zoonotic organisms should be monitored. In addition, the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) report annually on the incidence of these pathogens of zoonotic origin. However, the predominant wildlife disease monitoring system in Europe is through passive surveillance (Cardoso et al., 2021), which means that information is obtained or received by Veterinary Authorities without being actively sought. Each Member State has its routine monitoring protocols in animal health, food hygiene, and communicable human diseases, which in most cases do not contemplate wildlife (Cardoso et al., 2021). These states are required to notify any primary outbreaks of the diseases listed in Annex I of the Directive 2003/99/EC that are detected within their territory. Passive surveillance improves the probability of early detection of emerging diseases, but active surveillance (a survey carried out specifically for monitoring purposes) is essential to assess epidemiological dynamics and risk assessment of zoonoses and zoonotic agents to establish rapid alert systems (Cardoso et al., 2021; Chakraborty et al., 2022; Woods et al., 2019). For a correct risk assessment, active surveillance must be carried out methodically and regularly (Perez-Sancho et al., 2020). Moreover, without active surveillance, retrospective assessments of the origin of an epidemic or pandemic are complicated, hindering the decision-making process (Chakraborty et al., 2022).

Furthermore, active surveillance should be prioritised in areas with high population density, especially cities and peri-urban areas, where the spread of any contagious disease is more likely (Mackenstedt et al., 2015; Lindahl and Magnusson, 2020). Modern cities are in fact conurbations where people live in urban centres and surrounding towns intermingled with agrarian land, forest patches and parks, where people get in contact with many animal species, some of which may act as vector of zoonoses (Mackenstedt et al., 2015). In the large European cities, it is common for humans to coexist with animals, such as house sparrows, pigeons, storks, gulls, rats, rabbits, bats, *etc.*, and including in the last decades some invasive alien species such as parakeets or raccoons (Parrott et al., 2009). Some of these species may be present in high densities (especially rats, pigeons and parakeets), leading to a high probability of human contact with their faeces. In the case of invasive alien species, they may harbour a greater number of pathogens, as they often have weaker defence mechanisms against native parasites (Chinchio et al., 2020; Najberek et al., 2022) and, additionally, they may also carry parasites that are exotic to the region (Mori et al., 2018; Najberek et al., 2022). Furthermore, the prevalence of certain pathogens may be higher in urban animals due to higher exposures to these pathogens, especially to gastrointestinal ones (Rothenburger et al., 2017). However, there is a notable lack of research, and therefore knowledge, on zoonoses in urban and peri-urban environments (Rothenburger et al., 2017; Lindahl and Magnusson, 2020). Despite some species- and pathogen-specific studies (Camacho et al., 2016; Navarro et al., 2019; Perez-Sancho et al., 2020; Antilles et al., 2021), to our knowledge a broad overview of the potentially zoonotic organisms circulating in the urban ecosystem have not been provided.

Since active surveillance of several pathogens would be very expensive and time-consuming, an alternative option could be the use of next generation sequencing (NGS). Molecular approaches, such as metabarcoding, can provide detailed information on the organisms present in a sample through the massive sequencing of specific DNA fragments (Shokralla et al., 2012; Taberlet et al., 2012; Compson et al., 2020). Currently, metabarcoding is a relatively affordable and versatile technique that allows to quickly obtain information about the organisms of interest present in an environmental sample (Srivathsan et al., 2016; Cabodevilla et al., 2023). Using this technique and appropriate molecular markers, it should be possible to monitor zoonotic organisms of bacterial or eukaryotic origin (Razzauti et al., 2014; Jahan et al., 2021; Stensvold et al., 2021; Cabodevilla et al., 2024). In addition, this technique allows working with samples containing degraded DNA such as faecal remains (Srivathsan et al., 2016; Compson et al., 2020), thus facilitating sample collection and avoiding invasive techniques. Faecal samples (especially when fresh) can provide valuable information on organisms present in the host's intestinal tract (all faecal microbiota), including pathogens (Briscoe et al., 2022; Cabodevilla et al., 2023). In fact, it is extremely interesting to study the prevalence of pathogens in faeces, as faeces are the most common animal origin substance that humans come into contact with and one of the main sources of zoonoses (Slifko et al., 2000; García-Aljaro et al., 2019; Cabodevilla et al., 2024). As a drawback, it should be noted that metabarcoding only certifies the presence of the organism's DNA and not its viability or pathogenicity (Bulman et al., 2018). Even so, it could be an ideal tool for efficient screenings, providing information on potentially zoonotic organisms circulating in animals and their prevalence. Furthermore, the sequencing of the bacteria DNA present in faeces through metabarcoding allows for comprehensive information on the entire faecal microbiota. While this microbiota is not identical to the gut microbiota, it serves as a reliable proxy commonly used to study the gut microbiota in a non-invasive manner (Jandhyala et al., 2015). This is particularly significant because the gut microbiota of many species is not yet well-characterized, despite its crucial importance for their health (Lee and Hase, 2014; Jandhyala et al., 2015).

In the present work, we studied the presence and prevalence of potentially zoonotic pathogens in the urban wildlife of Madrid city (Spain) and its surroundings, one of the largest European cities with >3.2 million inhabitants and approximately 7 million including the entire metropolitan area (conurbation). Our main objective was to provide an overview of the potentially zoonotic organisms circulating and, with it, a better understanding of the potential risks. The study was carried out using metabarcoding of the 16S rRNA phylogenetic bacterial marker gene by analysing faeces from 9 species (6 birds and 3 mammals). In addition, using one of the species (European rabbit, *Oryctolagus cuniculus*; Linnaeus, 1758), whose habitat use is clearly ubiquitous (Gálvez-Bravo, 2017), we analysed the differences in the presence of these organisms between urban and rural areas. We hypothesize that in urban areas, wildlife, including rabbits, are more commonly exposed to pathogens due to their regular contact with anthropogenic waste, and consequently, they may have higher prevalence rates and potentially play an important role as vectors of these pathogens. We also aimed to explore whether metabarcoding could be a useful tool to monitor potentially zoonotic organisms circulating in the fauna of cities and their surroundings and to identify the most important reservoirs of each potentially zoonotic organism. Finally, we take advantage of the metabarcoding data obtained to provide insights into the faecal microbiota of the species.

2. Methods

2.1. Target species and sample collection

We collected faecal samples of 9 different urban and peri-urban species (six birds and three mammals; Table 1) that have frequent contact with people, either directly or indirectly (faeces). We collected samples of White stork (*Ciconia ciconia*; Linnaeus, 1758), Lesser Black-backed gull (*Larus fuscus*; Linnaeus, 1758), Rock dove (*Columba livia*; Gmelin, 1789), House sparrow (*Passer domesticus*; Linnaeus, 1758), Monk parakeet (*Myiopsitta monachus*; Boddaert, 1783), Rose-ringed parakeet (*Psittacula krameri*; Scopoli, 1769), Pipistrelle bat (*Pipistrellus sp.*; Kaup, 1829), European rabbit and Common raccoon (*Procyon lotor*; Linnaeus, 1758; Table 1; see abbreviated names used hereafter in Table 1). Storks and gulls are common in cities and usually feed on landfills (Cook et al., 2008; Gyimesi et al., 2016; Pineda-Pampliega et al., 2021). Stork samples (45) were collected in Colmenar Viejo (35 km North from Madrid city, Spain), very close to the Colmenar Viejo landfill (Supplementary Material Table A1). During winter, these storks sleep in the village, and spend the day eating at the landfill and its surroundings. Gull samples (45) were collected in Manzanares el Real (46 km North from Madrid city). These gulls feed mainly at the large landfills of Madrid (Colmenar Viejo and Valdemingómez, 15 km South from Madrid city), and roost at the Santillana reservoir, which supplies water to Madrid. Feral pigeons and sparrows are two iconic species of cities, which are abundant and commonly feed in parks and street pavements on anthropogenic waste. Samples of both species (66 samples of pigeon and 24 of sparrow) were collected in Madrid city (Spain) and surrounding towns (Supplementary Material Table A1). The two parakeet species are invasive alien species that colonised the city just a few decades ago (in the 80s). Moreover, Monk parakeet could be a key species as a reservoir of zoonotic pathogens due to its high density in Madrid, with around 40 % of the Spanish population (Molina et al., 2016), its colonial habits (Burger and Gochfeld, 2009; Rodríguez-Pastor et al., 2012), and its continuous contact with humans. Parakeet samples (80 samples of Monk parakeet and 10 of Rose-ringed parakeet) were provided by the Area de Gobierno de Medio Ambiente y Movilidad of the Madrid City Council. We included bats in the study because it is documented that bats can be reservoirs of different pathogens (Mühldorfer, 2013). Bat samples (16) were collected in Madrid city from bat boxes occupied by pipistrelles. The rabbit is a common mammal in urban parks and city outskirts, which has already been described as a reservoir of a zoonotic parasite (*Leishmania* sp.) (Jiménez et al., 2014), as well inhabits in purely rural areas. In the case of rabbits, we performed a stratified sampling in different locations of the Community of Madrid, categorising as urban (39) or rural (36) samples, based on proximity to a city/town and probability of contact with people and products of anthropic origin (estimated based on personal observations, areas with a high affluence of walkers, runners and bikers). Urban samples were collected within 350 m of towns or cities with a population of at least 10,000 inhabitants, while rural samples were taken from locations >450 m away, with an average distance >2 km. Two exceptions were made for two samples located 1 km from the city in areas with high pedestrian, runners, and bikers circulation, which were classified as urban due to their high potential for human contact. Finally, raccoons' samples (17) were provided by the Consejería de Medio Ambiente, Vivienda y Agricultura of the Community of Madrid. Raccoons are an important invasive species whose populations are continuously growing in Spain despite population control efforts (Valdez et al., 2022).

Table 1
Target species and their respective sample size after sample processing (number of samples with data after sequencing and filtering). More information about the samples' origin can be found at Supplementary Material Table A1.

	Common name	Scientific name	Name used in this work	Collected samples	Sample with data
Birds	White stork	*Ciconia ciconia*	Stork	45	37
	Lesser Black-backed gull	*Larus fuscus*	Gull	45	39
	Rock dove	*Columba livia*	Pigeon	66	61
	House sparrow	*Passer domesticus*	Sparrow	24	16
	Monk parakeet	*Myiopsitta monachus*	Monk parakeet	80	31
	Rose-ringed parakeet	*Psittacula krameri*	Rose-ringed parakeet	10	6
Mammals	Pipistrelle	*Pipistrellus* sp.	Bat	16	16
	European rabbit	*Oryctolagus cuniculus*	Urban Rabbit	39	31
	European rabbit	*Oryctolagus cuniculus*	Rural Rabbit	36	19
	Common raccoon	*Procyon lotor*	Raccoon	17	14

All faeces (Table 1), with the exception of samples from the two parakeet species and raccoon, were collected from the ground in roosting areas, and were collected as fresh as possible (still wet). In the case of the two parakeets and raccoon, samples were obtained by cloacal swabs. Since they are invasive exotic species, there is a population control program for both species lead by City Council and samples were facilitated by veterinarians conducting it. Faeces were collected in individual containers (Eppendorf or Falcons depending on the sample size) in 96° pure ethanol and stored cold until transfer to the laboratory, where they were kept frozen at −20 °C until DNA extraction (Vogtmann et al., 2017).

2.2. Sample processing

We used the MagBind Environmental DNA 96 Kit (Omega BioTek) to perform the DNA extractions. Then we checked the quality of extracted DNA through agarose gel electrophoresis and measured its concentration using Picogreen (Invitrogen). We used the primers 357Fw (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) targeting the V3-V4 region (Herlemann et al., 2011) for the amplification of the 16S rRNA bacterial phylogenetic marker, including Illumina sequencing adapters. For DNA amplification, we used 4 μl of

template DNA in a reaction mixture containing 35 μl of ddH2O, 2.75 μl of dNTPs (10 mM each), 11 μl of 5× reaction buffer, 0.3 μl of each primer (10 mM), and 0.6 μl of Q5® HighFidelity DNA Polymerase (New England Biolabs). Thermocycler conditions consisted of 95 °C for 30 s, followed by 20 cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s. PCR products were then treated with 20 U of ExoI (Thermo Scientific) for 20 min at 37 °C to remove the initial primers followed by 15 min at 80 °C to inactivate the enzyme. Then, samples were subjected to 10 more PCR cycles as above using primers bearing the required Illumina adapters and barcodes. We checked the amplicon libraries produced through agarose gel electrophoresis and measured their concentration using Picogreen (Invitrogen). We pooled equimolar amounts from each library, run on an agarose gel, excised the appropriate band, and purified using Monarch® DNA Gel Extraction Kit. Finally, the end product was sequenced with an Illumina MiSeq NGS platform using 600 cycle v3 reagent kits following the manufacturer's instructions.

2.3. Pathogens of interest: zoonoses and zoonotic agents

We consider pathogens of interest those mentioned by the European Centre for Disease Prevention and Control (ECDC) and/or the US Centers for Disease Control and Prevention (CDC) as zoonoses or zoonotic agents (Table 2; ECDC, 2022; CDC, 2022). In addition, we paid special attention to those zoonoses and zoonotic agents whose monitoring is mandatory in the European Union (Table 3), according to the European Parliament Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents. Although the information obtained by metabarcoding ensures the presence but not the viability and pathogenicity of the detected taxa, in this work we use this tool to quickly obtain an integrated overview on the zoonotic potential of the faeces of each species.

2.4. Bioinformatic and statistical analyses

We carried out all bioinformatic analyses using Cutadapt software (Martin, 2011) and R v4.1.2 software with the DADA2 v1.22.0 package (Callahan et al., 2016, 2017; R Core Team, 2021). First, we removed the primers from the sequences using Cutadapt. Then we filtered the sequences based on quality (allowing maximum of 2 expected errors) and trimmed based on sequences length (to 275 pb in forward and to 215 pb in reverse). Next, we merged forward and reverse sequences, constructed a table of amplicon sequence variants (hereafter ASV), and removed chimeric sequences. Finally, we assigned the taxonomy using a minBoot of 80 and SILVA (v138) reference database (Quast et al., 2012; Callahan et al., 2017; McLaren and Callahan, 2021). Once the ASV table was built, we performed a second data cleaning. We removed samples with <5000 reads, and among the remaining samples we removed ASVs not classified at phylum level or that presented <3 reads. We then calculated the relative read abundance (RRA) of each ASV for each sample and removed those ASVs with a maximum RRA lower than 0.01 %.

For the analysis of potential zoonoses, we only kept those ASVs that were identified at genus level as a genus considered zoonotic in Table 2. These ASVs were combined at the genus level for the following analyses. From this dataset, we have followed two different approaches. First, removing the samples of rural rabbits (keeping only urban samples), we characterized the prevalence of sequences of potential zoonotic agents in host species, with the aim of assessing the zoonotic potential of samples. We constructed a complete table of prevalence's and mean RRA values per genus in the host species. We also built a prevalence graph per potentially zoonotic genera, focusing only in those genera that have species whose monitoring is mandatory by the EU (The same information but grouped by host species can be found in Supplementary Material Fig. A1). On the other hand, using the data from rabbits (both urban

Table 3
Zoonoses and prokaryotic zoonotic agents listed in Annex 1 of Directive 2003/99/EC. Relevance = 1, zoonoses of mandatory monitoring in the European Union (Annex 1, Part A). Relevance = 2, zoonoses of mandatory monitoring depending on the epidemiological situation of the country (Annex 1, Part B)

Genus	Species	Relevance
Borrelia	*B. burgdorferi*	2
Brucella	*B.* sp.	1
Campylobacter	*C.* sp.	1
Chlamydia	*C. psittaci*	2
Clostridium	*C. botulinum*	1
Escherichia	*E. coli*[a]	1
Leptospira	*L.* sp.	2
Mycobacterium	*M. tuberculosis*	1
Mycobacterium	*M. bovis*	1
Mycobacterium	*M. caprae*	2
Listeria	*L. monocytogenes*	1
Salmonella	*S.* sp.	1
Vibrio	*V.* sp.	2
Yersinia	*Y.* sp.	2

[a] For *Escherichia coli* it is only compulsory to report cases of verotoxigenic *Escherichia coli*.

Table 2
Prokaryotic organisms listed as zoonotic agents by the European Centre for Disease Prevention and Control (ECDC; source = 1) and/or the US National Public Health Agency (CDC, source = 2).

Genus	Species	Source	Genus	Species	Source
Aeromonas	*Aeromonas* sp.	2	*Mycobacterium*	*M. marinum*	2
Bacillus	*Bacillus* sp.	1	*Mycobacterium*	*M. shottsii*	2
Bartonella	*Bartonella henselae*	2	*Mycobacterium*	*M. pseudoshottsii*	2
Borrelia	*Borrelia burgdorferi*	1 & 2	*Mycobacterium*	*M. fortuitum*	2
Brucella	*Brucella* sp.	1 & 2	*Mycobacterium*	*M. chelonae*	2
Campylobacter	*C.* sp.	1 & 2	*Mycobacterium*	*M. abscessus*	2
Capnocytophaga	*C.* sp.	2	*Mycobacterium*	*M. gordonae*	2
Chlamydia	*C. psittaci*	1 & 2	*Mycobacterium*	*M. salmoniphilum*	2
Clostridium	*C. botulinum*	1	*Mycobacterium*	*M. haemophilum*	2
Clostridium	*C. perfringens*	1	*Mycobacterium*	*M. tuberculosis*	1 & 2
Coxiella	*C. burnetii*	1 & 2	*Proteus*	*P. sp.*	1
Cronobacter	*C. sakazakii*	1	*Rickettsia*	*R. rickettsia*	2
Enterococcus	*E.* sp.	1	*Salmonella*	*S. sp.*	1 & 2
Erysipelothrix	*E. rhusiopathiae*	1 & 2	*Shigella*	*S. sp.*	1
Escherichia	*E. coli*	1 & 2	*Spirillum*	*S. minus*	2
Francisella	*F. tularensis*	1 & 2	*Staphylococcus*	*S. aureus*	1 & 2
Klebsiella	*K.* sp.	1	*Streptobacillus*	*S. moniliformis*	2
Leptospira	*L.* sp.	1 & 2	*Vibrio*	*V. sp.*	1
Listeria	*L. monocytogenes*	1	*Yersinia*	*Y. pestis*	1 & 2
Mycobacterium	*M. bovis*	1 & 2	*Yersinia*	*Y. enterocolitica*	1 & 2
Mycobacterium	*M. caprae*	1 & 2			

and rural) we performed a comparative analysis on the prevalence of potentially zoonotic organisms between urban and rural rabbits. To do this, we fitted General Linear Models (GLMs) with a binomial error and logit link function using the lme4 package in R (Bates et al., 2015). We fitted a model for each bacteria genera and used the rabbit sample category (urban or rural) as fixed factor.

Finally we carried out some analyses to characterise the faecal microbiota of the host species. First, we generated two heatmaps (one at Phylum level and another one at genus level) using the R package ampvis2 (Andersen et al., 2018). Subsequently, to assess the differences in faecal microbiota composition between host species, we carried out Nonmetric Multi-Dimensional Scaling (NMDS) and permutational multivariate analysis of variance (PERMANOVA) using the vegan v2.42 package in R (Dixon, 2003). Lastly, we analysed the relation between host species' faecal microbiotas by calculating the Pianka's index of overlap (Pianka, 1973) using the R package EcoSimR (Gotelli et al., 2015). This index ranges from 0 to 1 (full overlap) and allows to quantify the degree of similarity between two samples, faecal microbiotas in our case. We used the R package corrplot (Wei and Simko, 2021) to visualize these values.

3. Results

During the filtering process, 29 samples were excluded for having fewer than 5000 reads, resulting in 270 samples retained from the original 299 with sequencing data. After all filtering, an average coverage per sample of 25,737.2 reads (maximum of 175,480 and minimum of 5037) and a total of 13,534 ASVs were obtained. 51.3 % of these ASVs could be identified to genus level, grouped into 777 genera.

3.1. Potentially zoonotic pathogens

Among the prokaryotic organisms classified as zoonotic (Table 2), we detected DNA of 21 potentially zoonotic bacterial genera in the analysed samples (Supplementary Material Table A2), 10 of which include zoonotic species of mandatory monitoring in the European Union (Fig. 1, Table 3): *Brucella*, *Campylobacter*, *Chlamydia*, *Clostridium*, *Escherichia*, *Listeria*, *Mycobacterium*, *Salmonella*, *Vibrio*, and *Yersinia*. *Clostridium* and *Escherichia* were detected in all host species, with prevalence higher than 0.40 for all of them except for the rabbit (Fig. 1). *Brucella* was only detected in pigeon and bat, with very low prevalence (proportion of samples) in pigeon (0.02) but with a prevalence of 0.14 in bat faeces (Fig. 1; Supplementary Information Table A2). *Campylobacter* was detected in 7 of the 9 host species, with prevalence over 0.50 in sparrow (0.50) and urban rabbit (0.55) and prevalence over 0.10 in Rose-ringed parakeet (0.17), Monk parakeet (0.16), and pigeon (0.11). *Chlamydia* was only detected in bats, with a prevalence of 0.48. *Listeria* was detected in 6 species. *Mycobacterium* was detected in 8 out of the 9 species, with prevalence higher than 0.10 in all of them except for the stork and the Monk parakeet. *Salmonella* was only detected in a stork samples. *Vibrio* was detected in bats and raccoon. And *Yersinia* was only detected in sparrow, with a prevalence of 0.29 (Fig. 1, Supplementary Information Table A2). Overall, the bat was the host species in which the most potentially zoonotic genera of mandatory monitoring were detected, 7 out of 10 (Fig. 1; Supplementary Material Fig. A1), followed by stork, pigeons and sparrows with 6 genera detected.

In addition, the taxonomic assignment (using a minBoot threshold of 80) enabled species-level identification for 37 % of the ASVs categorized as potentially zoonotic genera of mandatory monitoring, with 10 zoonotic species of relevance identified (Table 4): *Brucella melitensis*, *Campylobacter cuniculorum*, *Campylobacter jejuni*, *Campylobacter lari*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, *Vibrio cholerae*, *Yersinia enterocolitica* and *Yersinia rohdei*. *Brucella melitensis* was detected in pigeon (0.02) and bat (0.18). *Campylobacter cuniculorum* was detected in Monk parakeet and urban rabbit, in the latter with a prevalence of 0.42. *Campylobacter jejuni* was identified in faeces of pigeon and sparrow. *Escherichia coli* was detected in all host species and in most cases with high prevalence. However, in the case of *Escherichia coli* it could be either zoonotic or risk-free as our analyses does not allow us to discriminate between them. *Listeria monocytogenes* was present in samples of stork, gull, pigeon, sparrow, Monk parakeet, and bat, with highest prevalence in gull (0.10), but highest abundance per sample (RRA) in sparrow (with up to 0.68 % of reads) and Monk parakeet (with up to 0.51 % of reads; Table 4). *Salmonella enterica* was only detected in a stork sample and *Vibrio cholerae* was only detected in a single bat sample. Finally, both *Yersinia* species were detected in sparrow.

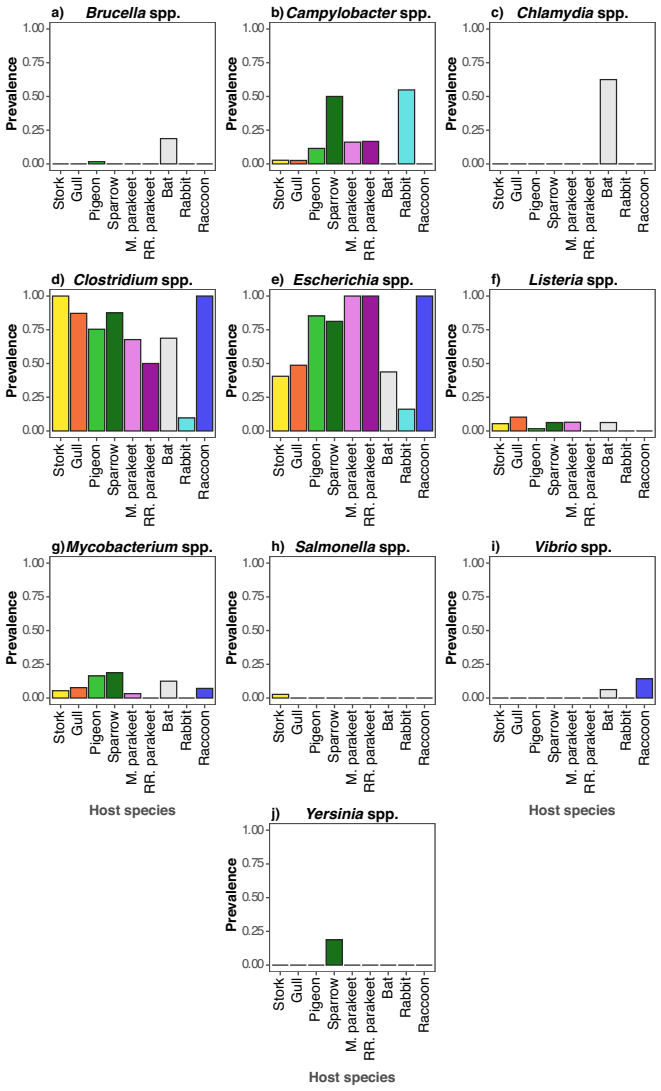


Fig. 1. Prevalence of each zoonotic bacterial genus that is mandatory for monitoring in the EU in the faeces of host species. We have used the abbreviation M. parakeet to refer to Monk parakeet and RR. parakeet to refer to Rose-ringed parakeet.

3.2. Differences between urban and rural rabbits in the prevalence of zoonotic pathogens

Regarding the differences on the prevalence of potentially zoonotic pathogens between urban and rural rabbits, our results showed a significant effect for two bacterial genera (Fig. 2; Supplementary Information Table A3), *Campylobacter* ($F_{1,48} = 10.50$, $P < 0.01$) and *Staphylococcus* ($F_{1,48} = 4.62$, $P < 0.05$). In both cases, urban rabbits showed higher prevalence than rural rabbits. For *Campylobacter* (of mandatory monitoring), urban rabbits had a prevalence of 0.55 while

Table 4
Prevalence of ASVs identified as potentially zoonotic bacteria species of mandatory monitoring. In brackets and grey the maximum RRA value is given (Relative read abundance in percentage obtained in a single sample). Many ASVs could not be identified to specie level, so this table provides only partial data.

	Stork	Gull	Pigeon	Sparrow	Monk parakeet	Rose-ringed parakeet	Bat	Urban Rabbit	Rural Rabbit	Raccoon
Brucella melitensis			0.02 (0.01)				0.18 (0.11)			
Campylobacter cuniculorum					0.06 (0.02)			0.42 (0.48)		
Campylobacter jejuni			0.03 (0.07)	0.06 (0.09)						
Campylobacter lari	0.03 (0.02)									
Escherichia coli	0.41 (4.01)	0.49 (5.52)	0.84 (49.93)	0.81 (13.32)	1 (95.55)	1 (4.53)	0.44 (4.80)	0.16 (0.21)	0.16 (0.02)	1 (50.60)
Listeria monocytogenes	0.05 (0.05)	0.10 (0.06)	0.02 (0.05)	0.06 (0.68)	0.06 (0.51)		0.06 (0.20)			
Salmonella enterica	0.03 (0.04)									
Vibrio cholerae							0.06 (0.01)			
Yersinia enterocolitica				0.13 (2.89)						
Yersinia rohdei				0.06 (0.05)						

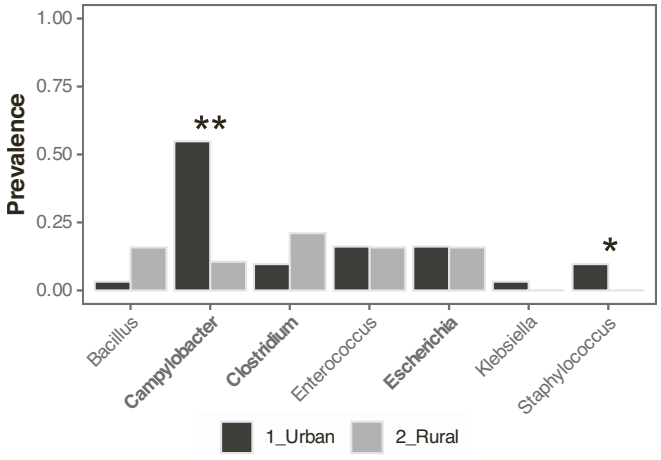


Fig. 2. Differences on the prevalence of potentially zoonotic bacterial genera between urban and rural rabbits. Bacterial genera of mandatory monitoring in the EU are highlighted in bold. $^{**}P < 0.01$; $^{*}P < 0.05$.

rural rabbits had a prevalence of only 0.11 (Fig. 2; Supplementary Information Table A3).

3.3. Faecal microbiota

According to our results, the faecal microbiota of all studied host species, both birds and mammals, was mainly composed of bacteria of the phylum Firmicutes (Supplementary Material Fig. A2). The phylum Proteobacteria was also important (>10 %) in the microbiota of pigeon, sparrow, Monk parakeet, bat and raccoon (Supplementary Material Fig. A2).

Regarding the main bacterial genera detected in their faecal microbiota, we found important differences among host species (Fig. 3), being quite pronounced in birds, and especially between native and invasive species (Fig. 3). The faecal microbiota of native birds was mainly composed of the genus *Lactobacillus* (RRA above 50 % in stork, pigeon and sparrow) and also had a higher RRA of *Streptococcus* and *Catellicoccus* genera than invasive birds (Fig. 3). In contrast, parakeets had higher RRA of *Enterococcus* genus than native species and Monk parakeet a 42 % RRA of *Escherichia*. The faecal microbiota of bats consisted mainly of *Enterococcus*, *Lactococcus* and *Staphylococcus* genera. Rabbit's faecal microbiota had some similarity to that of the Rose-ringed parakeet, with >10 % RRA of *Akkermansia*, *Monoglobus*, *Ruminococcus* and *Tyzzerella* genera. Finally, the faecal microbiota of the raccoon was mainly composed of *Clostidium* and *Escherichia* genera (Fig. 3). The NMDS and PERMANOVA analyses confirmed significant differences between the faecal microbiota of native and invasive birds (parakeets), with the latter clustering closer to rabbits and raccoons than to native bird species (Fig. 4). Native birds were grouped closer together, especially storks, gulls and sparrows, showing the highest similarity in faecal microbiota (Fig. 4). The correlation plot showed the same relationship (Supplementary Information Fig. A3), with an important overlap in the faecal microbiota composition of the four native bird species (>0.6 out of 1) and very little overlap between parakeets and native birds. Surprisingly Rose-ringed parakeet showed a high overlap with the rabbit (0.79 out of 1; Supplementary Information Fig. A3).

4. Discussion

Our study provide a broad picture of the potentially zoonotic bacteria circulating in urban species of a large European city and their prevalence, as well as a better understanding of the risk that interactions with these animals and their faeces may pose. We have detected potentially zoonotic bacterial genera in all the host species, 10 of these genera with zoonotic species of mandatory monitoring in the European Union. Besides, our results demonstrate that urban rabbits have a higher prevalence of certain pathogens, with a prevalence of >0.5 of *Campylobacter* compared to the prevalence of 0.1 in rural rabbits. Moreover, our results indicate that metabarcoding of faecal samples is a useful tool to perform a first screening of the scenario, providing information on potentially zoonotic organisms circulating in vertebrates in contact with human and their prevalence, as well as, to identify the most important reservoirs of each potentially zoonotic organism. Finally, we also provide detailed information on the composition of the faecal microbiota of studied host species.

4.1. Potentially zoonotic pathogens

An outstanding result of this study is the detection of DNA from potentially zoonotic bacterial genera in all host species. Among the 21 potentially zoonotic genera detected, there are 10 which have zoonotic species of mandatory monitoring in the European Union. These ten were detected in several host species, which could be due to their trophic ecology, including in their diet remains of food of anthropic origin (Cook et al., 2008; Gyimesi et al., 2016; Pineda-Pampliega et al., 2021; Postigo et al., 2021; Mazzoni et al., 2022). The two most common and abundant genera were *Clostridium* and *Escherichia*, being detected in all host species. Both genera can be a natural part of the host' faecal microbiota (Silva et al., 2012; Grond et al., 2018). However, it should be noted that over half of the *Clostridium*-affiliated reads were identified as *C. perfringens*, a species with pathogenic strains (Songer, 2010; Grond

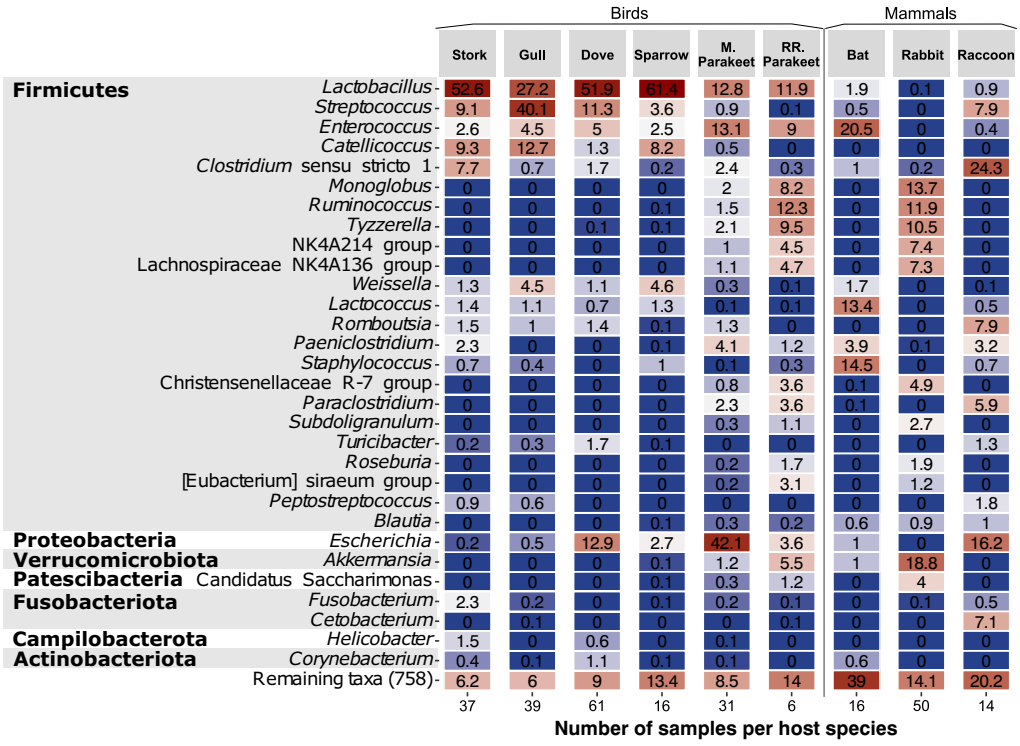


Fig. 3. Heatmap by host species with the 30 most abundant (RRA) bacterial genus detected. We have used the abbreviation M. parakeet to refer to Monk parakeet and RR. parakeet to refer to Rose-ringed parakeet.

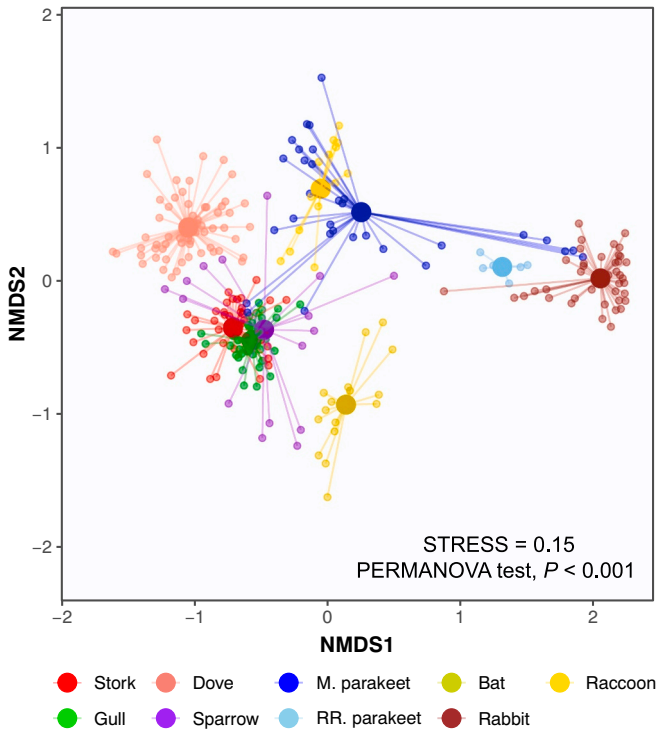


Fig. 4. Graphical representation of the similarity between hosts' faecal microbiota obtained from the NMDS analysis based on the ASVs. The large symbols represent the centroid for each species, while the small dots represent the data for each of the samples per species. We have used the abbreviation M. parakeet to refer to Monk parakeet and RR. parakeet to refer to Rose-ringed parakeet.

et al., 2018). Unfortunately, metabarcoding, at least with these primers and reference database, does not allow to obtain precise information about the pathogenic capacity of detected *C. perfringens* and *Escherichia coli*, as it only provides information about the presence of the organism and not about its strain. In future studies this should be complemented with the use of other primers specific to pathogenic strains.

In addition to these two genera, other potentially zoonotic genera such as, *Brucella*, *Campylobacter*, *Chlamydia*, *Listeria*, *Mycobacterium*, *Salmonella*, *Vibrio*, and *Yersinia* were detected. Among these, *Mycobacterium*, *Campylobacter* and *Listeria* were the most widespread, detected in 7, 7 and 6 host species respectively. In this case, all ASVs of the genus *Mycobacterium* identified to species level do not appear to be zoonotic, as the species identified have not been reported infecting humans. Detected *Campylobacter* and *Listeria*, on the other hand, are of great health relevance (Chlebicz and Śliżewska, 2018). *Campylobacter* has already been commonly described in birds as a zoonotic agent (Grond et al., 2018; Perez-Sancho et al., 2020; Antilles et al., 2021) and in our case it was found in all bird species, with the highest prevalence in sparrow, being detected in the half of the sparrow's samples. We found it also in >50 % of urban rabbit's samples, and in >10 % of Rose-ringed parakeet's, Monk parakeet's and pigeon's samples. Moreover, the highest abundance (RRA) of *Campylobacter* genus was found in pigeons and sparrows. In these two species *Campylobacter jejuni* was detected, the zoonotic agent responsible for most Campylobacteriosis in humans (Chlebicz and Śliżewska, 2018).

Therefore, contact with faeces from any species of urban bird or rabbit could pose a zoonotic risk of Campylobacteriosis, but especially sparrow and pigeon faeces, two very common species that are easy to come into contact with. *Listeria* genus has not been studied as much as *Campylobacter* in birds, but it is also an important zoonotic agent (Chlebicz and Śliżewska, 2018). We found it in almost all urban bird species, having not been detected only in the Rose-ringed parakeet. In this case, the highest prevalence was found in gulls (10 % of samples), although the highest read abundance was found in the sparrow followed by the Monk parakeet. All of the *Listeria* detected were classified as

Listeria monocytogenes, the zoonotic bacteria responsible of listeriosis in humans (Chlebicz and Śliżewska, 2018), although our approach does not allow the determination of the strain and therefore its pathogenicity.

The remaining zoonotic organisms of mandatory monitoring were detected in specific species. *Brucella* genus was detected in pigeon and bat. *Chlamydia* genus was detected only in bat samples, detected in 10 out of 16 samples. It should be noted that in this study no *Chlamydia* was detected in any bird, not even in parakeets. *Salmonella* was detected in a single stork sample and *Vibrio* was detected in two species (bat, and raccoon), identifying *Vibrio cholerae*'s DNA in a bat sample. Finally, *Yersinia* was only detected in sparrow samples, identified as *Y. rohdei* and *Y. enterocolitica*; the zoonotic bacteria responsible of yersiniosis in humans (Chlebicz and Śliżewska, 2018).

Overall, bats appear to be the species of greatest importance as a reservoir of zoonotic organisms, having detected in them 7 out of the 10 species of mandatory monitoring, including *Vibrio cholerae*. Besides, the bird species with the highest prevalence and relative abundance of zoonotic organisms was the sparrow, including *Campylobacter jejuni*, *Listeria monocytogenes*, and *Yersinia enterocolitica*. Given their ecology, their proximity to humans, and their abundance in large cities, the pigeon would be the second species potentially posing the greatest risk after the sparrow. Pigeon usually form large groups in cities, they roost in groups or colonies (Stukenholtz et al., 2019) and they feed in public spaces where it is easy to come into contact with their fresh faeces due to their size and availability. All this facilitates pathogen spread. However, to accurately determine the risk posed by each species, it would be necessary to consider their density, potential population increases, and likelihood of contact with humans. Unfortunately, we do not have detailed information on densities in the study area. But because of their habitat preferences and adaptability, sparrows and pigeons likely have the closest contact with humans in Madrid.

Furthermore, in the case of the stork and the gull, although we have found lower prevalence of potentially zoonotic bacteria on them, the fact that they regularly feed in landfills might lead to a higher pathogenic potential of these bacteria. It should be noted that landfills provide conditions for the development and transmission of antibiotic resistant pathogens (Martín-Maldonado et al., 2020), so that the species that frequent them could be reservoirs of several particularly dangerous zoonotic pathogens. In fact, according to Martín-Maldonado et al. (2020) 40.5 % of *Salmonella* found in storks feeding in landfills are resistant to some antibiotic. Another study has shown that storks feeding in landfills have a higher number of multi-resistant *Escherichia coli* (Pineda-Pampliega et al., 2021).

Regarding the comparison between urban and rural rabbits, we found a significantly higher prevalence of *Campylobacter* and *Staphylococcus* in urban ones. This could be because urban rabbits, as other urban animals, are more frequently exposed to these pathogens due to their regular contact with anthropogenic waste (Rothenburger et al., 2017). However, this could also be related to other reasons, such as their density, as they might reach higher densities in cities (Ziege et al., 2016). In any case, this result supports the evidence that urban wildlife tends to have higher prevalence of pathogens, leading to more opportunities for pathogen transmission. This being the case, urban fauna could play an important role as reservoirs and dispersers of these pathogens, an issue of potential concern for initiatives to improve the presence of biodiversity in urbanized areas, like the EU Green infrastructure (Maes et al., 2015; European Commission, 2022). However, there is still a lack of knowledge in this respect, and more research is needed to verify that faeces from urban wildlife have a higher zoonotic risk than faeces from rural wildlife.

4.2. Faecal microbiota

As was previously described in wildlife (Grond et al., 2018), our results showed that the faecal microbiota of the different host species was mainly composed of the phylum Firmicutes, with an important presence of the phyla Proteobacteria and Actinobacteriota in some of them. However, we observed important differences in the composition of the faecal microbiota between host species. In birds, we found a greater similarity within native urban species compared to parakeets (invasive species). It is known that birds' gut microbiota is highly dependent on diet, but also on host phylogeny, habitat, nest environment and season (Grond et al., 2018; Matheen et al., 2022). The strong similarities among native urban bird species could hardly be explained by diet, as there are clear differences in the trophic habits between them. While stork and gull are mainly carnivorous (Gyimesi et al., 2016; Chenchouni, 2017), pigeon and sparrow are mainly granivorous (Murton and Westwood, 1966; Whelan et al., 2015). On the other hand, the differences with parakeets could be related to various aspects. In this case, the dietary difference could be an explanation, as although parakeets could feed regularly on waste of anthropogenic origin inside the city their diet consists mainly of vegetal material (Mazzoni et al., 2022). Another explanation could be the fact that native species are better adapted to their environment unlike alien species. These differences could be also related to the biogeographical origin of the different host species, being the native species from Europe while Monk parakeet is originally from South America and Rose-ringed parakeet from Africa and Asia. Besides, there does not seem to be a link with their phylogenetic origin, as the native urban species are also phylogenetically distant species (Prum et al., 2015). However, we also cannot rule out the possibility that these differences could be related to the type of sample (Grond et al., 2018; Matheen et al., 2022), since in the native urban species faeces were collected as fresh droppings and in the parakeets the samples were collected by cloacal swab directly from the intestinal tract.

Regarding mammals, we found large differences in faecal microbiota between them, with extremely low Pianka's overlapping values. This is probably due to two main factors. First, all three have very different diets; the studied bats are strictly insectivorous (Alberdi et al., 2020), rabbits are strictly herbivorous (Martins et al., 2002), and raccoons are omnivorous (Rulison et al., 2012), and second, they are species from very distant phylogenetic groups (Prasad, et al., 2008). Surprisingly, a high degree (0.79 out of 1) of overlap in faecal microbiota composition was observed between rabbit and Rose-ringed parakeet. We found no apparent explanation for this similarity, as if it were related to diet, it would be expected to find the same relationship with the Monk parakeet.

5. Conclusion

In conclusion, our results provided a broad picture of the potentially zoonotic bacteria circulating in wildlife of a large European city, providing information on the presence and prevalence of several organisms. This information highlights the risk that coming into contact with fresh urban wildlife faeces could pose, as we also found that urban wildlife has a higher prevalence of these microorganisms. Urban birds and bats were the species that pose the highest risk, with *Campylobacter* and *Listeria* in birds and *Chlamydia* and *V. cholerae* in bats as the most important potentially zoonotic pathogens. Taking into account their ecology, possibly the most important species from a health point of view are sparrow, pigeon and bat. As the methodology used does not allow us to be sure that the detected DNA comes from viable cells, in future studies it would be very important to carry out specific monitoring of the pathogens present in urban bat populations and in particular it would be advisable to verify the presence of *Vibrio cholerae* in this species so important in the ecosystem (Kunz et al., 2011). Moreover, our results show that, with limitations (especially the lack of strain-level resolution), metabarcoding provides highly relevant information at a relatively low cost and effort, and can be used to quickly obtain rapid screenings, providing information on potentially zoonotic organisms and their prevalence, as well as identifying the most important reservoirs. This information makes it much easier to focus future monitoring efforts on the most relevant pathogens and host species.

CRediT authorship contribution statement

Xabier Cabodevilla: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Juan E. Malo:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Daniel Aguirre de Cárcer:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Julia Zurdo:** Writing – review & editing, Resources, Investigation, Conceptualization. **Rubén Chaboy-Cansado:** Writing – review & editing, Investigation. **Alberto Rastrojo:** Writing – review & editing, Investigation. **Francisco J. García:** Writing – review & editing, Resources. **Juan Traba:** Writing – review & editing, Resources, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Juan Traba reports financial support was provided by Community of Madrid. Juan Traba reports article publishing charges was provided by Community of Madrid. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Authors are especially grateful to the Área de Medioambiente y Movilidad (Departamento de Fauna y Biodiversidad) from the city council of Madrid, and to the Centre for the Recovery of Wild Animals (CRAS, Área de Conservación de Flora y Fauna) from the Consejería de Medio Ambiente, Vivienda y Agricultura of the Regional Government of Madrid for providing parakeet and raccoon samples. Francisco Sobrino Castelló, Juan Carlos Ortíz and José Luis Menéndez collaborated in sample collection. This study was funded by the project COVTRAVI-19-CM funded by the Comunidad de Madrid through FEDER and within the framework of the Recovery, Transformation and Resiliency Plan (European Union). This paper contributes to project REMEDINAL TE-CM (P2018/ EMT4338) from Regional Government of Madrid. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.175866.

References

Alberdi, A., Razgour, O., Aizpurua, O., Novella-Fernandez, R., Aihartza, J., Budinski, I., Garin, I., Ibáñez, C., Izagirre, E., Rebelo, H., Russo, D., Vlaschenko, A., Zhelyazkova, V., Zrnčić, V., Gilbert, M.T.P., 2020. DNA metabarcoding and spatial modelling link diet diversification with distribution homogeneity in European bats. Nat. Commun. 11 (1), 1154. https://doi.org/10.1038/s41467-020-14961-2.

Andersen, K.S., Kirkegaard, R.H., Karst, S.M., Albertsen, M., 2018. ampvis2: an R package to analyse and visualise 16S rRNA amplicon data. BioRxiv, 299537. https://doi.org/10.1101/299537.

Antilles, N., García-Bocanegra, I., Alba-Casals, A., López-Soria, S., Pérez-Méndez, N., Saco, M., González-Solís, J., Cerdà-Cuéllar, M., 2021. Occurrence and antimicrobial resistance of zoonotic enteropathogens in gulls from southern Europe. Sci. Total Environ. 763, 143018 https://doi.org/10.1016/j.scitotenv.2020.143018.

Bates, D., Maechler, M., Bolker, B., Walker, S., Christensen, R.H.B., Singmann, H., Bolker, M.B., 2015. Package 'lme4'. Convergence 12 (1), 2.

Briscoe, A.G., Nichols, S., Hartikainen, H., Knipe, H., Foster, R., Green, A.J., Okamura, B., Bass, D., 2022. High-throughput sequencing of faeces provides evidence for dispersal of parasites and pathogens by migratory waterbirds. Mol. Ecol. Resour. 22 (4), 1303–1318. https://doi.org/10.1111/1755-0998.13548.

Bulman, S.R., McDougal, R.L., Hill, K., Lear, G., 2018. Opportunities and limitations for DNA metabarcoding in Australasian plant-pathogen biosecurity. Australas. Plant Pathol. 47, 467–474. https://doi.org/10.1007/s13313-018-0579-3.

Burger, J., Gochfeld, M., 2009. Exotic monk parakeets (*Myiopsitta monachus*) in New Jersey: nest site selection, rebuilding following removal, and their urban wildlife appeal. Urban Ecosyst. 12 (2), 185196 https://doi.org/10.1007/s11252-009-0094-y.

Cabodevilla, X., Gómez-Moliner, B.J., Abad, N., Madeira, M.J., 2023. Simultaneous analysis of the intestinal parasites and diet through eDNA metabarcoding. Integr. Zool. 18 (3), 399–413. https://doi.org/10.1111/1749-4877.12634.

Cabodevilla, X., Malo, J.E., Aguirre de Carcer, D., Zurdo, J., Chaboy-Cansado, R., Rastrojo, A., Traba, J., 2024. DNA prevalence of eukaryotic parasites with zoonotic potential in urban-associated birds. Birds 5 (3), 375–387. https://doi.org/10.3390/birds5030025.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: Highresolution sample inference from Illumina amplicon data. Nat. Methods 13 (7), 581583. https://doi.org/10.1038/nmeth.3869.

Callahan, B.J., McMurdie, P.J., Holmes, S.P., 2017. Exact sequence variants should replace operational taxonomic units in markergene data analysis. ISME J. 11 (12), 26392643 https://doi.org/10.1038/ismej.2017.119 (2017/12/01).

Camacho, M., Hernández, J.M., Lima-Barbero, J.F., Höfle, U., 2016. Use of wildlife rehabilitation centres in pathogen surveillance: a case study in white storks (*Ciconia ciconia*). Prev. Vet. Med. 130, 106–111. https://doi.org/10.1016/j.prevetmed.2016.06.012.

Cardoso, B., GarcíaBocanegra, I., Acevedo, P., Cáceres, G., Alves, P.C., Gortázar, C., 2021. Stepping up from wildlife disease surveillance to integrated wildlife monitoring in Europe. Res. J. Vet. Sci. 144, 149156 https://doi.org/10.1016/j.rvsc.2021.11.003.

Carlson, C.J., Farrell, M.J., Grange, Z., Han, B.A., Mollentze, N., Phelan, A.L., Webala, P. W., 2021. The future of zoonotic risk prediction. Philos. Trans. R. Soc. B 376 (1837), 20200358. https://doi.org/10.1098/rstb.2020.0358.

CDC, 2022. U.S. Centers for Disease Control and Prevention. https://www.cdc.gov. (Accessed 17 November 2022).

Chakraborty, C., Bhattacharya, M., Nandi, S.S., Mohapatra, R.K., Dhama, K., Agoramoorthy, G., 2022. Appearance and reappearance of zoonotic disease during the pandemic period: Longterm monitoring and analysis of zoonosis is crucial to confirm the animal origin of SARSCoV2 and monkeypox virus. Vet. Q. 111 https://doi.org/10.1080/01652176.2022.2086718.

Chenchouni, H., 2017. Variation in White Stork (*Ciconia ciconia*) diet along a climatic gradient and across rural-to-urban landscapes in North Africa. Int. J. Biometeorol. 61 (3), 549–564. https://doi.org/10.1007/s00484-016-1232-x.

Chinchio, E., Crotta, M., Romeo, C., Drewe, J.A., Guitian, J., Ferrari, N., 2020. Invasive alien species and disease risk: an open challenge in public and animal health. PLoS Pathog. 16 (10), e1008922 https://doi.org/10.1371/journal.ppat.1008922.

Chlebicz, A., Śliżewska, K., 2018. Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: a review. Int. J. Environ. Res. Public Health 15 (5), 863. https://doi.org/10.3390/ijerph15050863.

Compson, Z.G., McClenaghan, B., Singer, G.A., Fahner, N.A., Hajibabaei, M., 2020. Metabarcoding from microbes to mammals: comprehensive bioassessment on a global scale. Front. Ecol. Evol. 8, 581835 https://doi.org/10.3389/fevo.2020.581835.

Cook, A., Rushton, S., Allan, J., Baxter, A., 2008. An evaluation of techniques to control problem bird species on landfill sites. Environ. Manag. 41 (6), 834843 https://doi.org/10.1007/s00267-008-9077-7.

Dixon, P., 2003. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14 (6), 927930 https://doi.org/10.1111/j.16541103.2003.tb02228.x.

ECDC, 2022. European Centre for Disease Prevention and Control. https://www.ecdc.europa.eu/en. (Accessed 17 November 2022).

European Commission, 2022. Guidance on a Strategic Framework for Further Supporting the Deployment of EU-Level Green and Blue Infrastructure. European Commission, Brussel, Belgium, p. 101. Available online: https://ec.europa.eu/environment/nature/ecosystems/pdf/SWD_2019_193_F1_STAFF_WORKING_PAPER_EN_V4_P1_1024680.PDF. (Accessed 22 May 2024).

Gálvez-Bravo, L., 2017. Conejo – *Oryctolagus cuniculus*. In: Salvador, A., Barja, I. (Eds.), Enciclopedia Virtual de los Vertebrados Españoles. Museo Nacional de Ciencias Naturales, Madrid. http://www.vertebradosibericos.org/.

García-Aljaro, C., Blanch, A.R., Campos, C., Jofre, J., Lucena, F., 2019. Pathogens, faecal indicators and human-specific microbial source-tracking markers in sewage. J. Appl. Microbiol. 126 (3), 701–717. https://doi.org/10.1111/jam.14112.

Gotelli, N.J., Hart, E.M., Ellison, A.M., 2015. EcoSimR: null model analysis for ecological data. R package version 0.1.0. http://github.com/gotellilab/EcoSimR.

Grond, K., Sandercock, B.K., Jumpponen, A., Zeglin, L.H., 2018. The avian gut microbiota: community, physiology and function in wild birds. J. Avian Biol. 49 (11), e01788 https://doi.org/10.1111/jav.01788.

Gyimesi, A., Boudewijn, T.J., Buijs, R.J., ShamounBaranes, J.Z., de Jong, J.W., Fijn, R.C., Poot, M.J., 2016. Lesser blackbacked gulls *Larus fuscus* thriving on a nonmarine diet. Bird Study 63 (2), 241249. https://doi.org/10.1080/00063657.2016.1180341.

Herlemann, D.P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J.J., Andersson, A.F., 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. ISME J. 5 (10), 15711579 https://doi.org/10.1038/ismej.2011.41.

Jahan, N.A., Lindsey, L.L., Kipp, E.J., Reinschmidt, A., Heins, B.J., Runck, A.M., Larsen, P.A., 2021. Nanopore-based surveillance of zoonotic bacterial pathogens in farm-dwelling peridomestic rodents. Pathogens 10 (9), 1183. https://doi.org/10.3390/pathogens10091183.

Jandhyala, S.M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., Reddy, D.N., 2015. Role of the normal gut microbiota. World J. Gastroenterol. 21 (29), 8787. https://doi.org/10.3748/wjg.v21.i29.8787.

Jiménez, M., González, E., MartínMartín, I., Hernández, S., Molina, R., 2014. Could wild rabbits (*Oryctolagus cuniculus*) be reservoirs for Leishmania infantum in the focus of Madrid, Spain? Vet. Parasitol. 202 (34), 296300 https://doi.org/10.1016/j.vetpar.2014.03.027.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., Daszak, P., 2008. Global trends in emerging infectious diseases. Nature 451 (7181), 990993. https://doi.org/10.1038/nature06536.

Kunz, T.H., Braun de Torrez, E., Bauer, D., Lobova, T., Fleming, T.H., 2011. Ecosystem services provided by bats. Ann. N. Y. Acad. Sci. 1223 (1), 1–38. https://doi.org/10.1111/j.1749-6632.2011.06004.x.

Lee, W.J., Hase, K., 2014. Gut microbiota–generated metabolites in animal health and disease. Nat. Chem. Biol. 10 (6), 416–424. https://doi.org/10.1038/nchembio.1535.

Lindahl, J., Magnusson, U., 2020. Zoonotic pathogens in urban animals: enough research to protect the health of the urban population? Anim. Health Res. Rev. 21 (1), 50–60. https://doi.org/10.1017/S1466252319000100.

Mackenstedt, U., Jenkins, D., Romig, T., 2015. The role of wildlife in the transmission of parasitic zoonoses in peri-urban and urban areas. Int. J. Parasitol. Parasites Wildl. 4 (1), 71–79. https://doi.org/10.1016/j.ijppaw.2015.01.006.

Maes, J., Barbosa, A., Baranzelli, C., Zulian, G., Batista e Silva, F., Vandecasteele, I., Lavalle, C., 2015. More green infrastructure is required to maintain ecosystem services under current trends in land-use change in Europe. Landsc. Ecol. 30, 517–534. https://doi.org/10.1007/s10980-014-0083-2.

Magouras, I., Brookes, V.J., Jori, F., Martin, A., Pfeiffer, D.U., Dürr, S., 2020. Emerging zoonotic diseases: should we rethink the animal–human interface? Front. Vet. Sci. 7, 582743 https://doi.org/10.3389/fvets.2020.582743.

Martin, M., 2011. Cutadapt removes adapter sequences from highthroughput sequencing reads. EMBnet J. 17 (1), 1012. https://doi.org/10.14806/ej.17.1.200.

Martín-Maldonado, B., Vega, S., MencíaGutiérrez, A., LorenzoRebenaque, L., De Frutos, C., González, F., Marin, C., 2020. Urban birds: an important source of antimicrobial resistant Salmonella strains in Central Spain. Comp. Immunol. Microbiol. Infect. Dis. 72, 101519 https://doi.org/10.1016/j.cimid.2020.101519.

Martins, H., Milne, J.A., Rego, F., 2002. Seasonal and spatial variation in the diet of the wild rabbit (*Oryctolagus cuniculus* L.) in Portugal. J. Zool. 258 (3), 395–404. https://doi.org/10.1017/S0952836902001541.

Matheen, M.I.A., Gillings, M.R., Dudaniec, R.Y., 2022. Dominant factors shaping the gut microbiota of wild birds. Emu 122 (3–4), 255–268. https://doi.org/10.1080/01584197.2022.2114088.

Mazzoni, D., Pascual, J., Arroyo, L., Montalvo, T., González-Solís, J., Senar, J.C., 2022. The diet of Monk Parakeet *Myiopsitta monachus* nestlings in an urban area: a study using stable isotopes. Bird Study 68 (4), 455–461. https://doi.org/10.1080/00063657.2022.2113856.

McLaren, M.R., Callahan, B.J., 2021. Silva 138.1 Prokaryotic SSU Taxonomic Training Data Formatted for DADA2 [Data Set]. Zenodo. https://doi.org/10.5281/zenodo.4587955.

Molina, B., Postigo, J.L., Muñoz, A.R., Del Moral, J.C., 2016. La cotorra argentina en España, población reproductora en 2015 y método de censo. SEO/BirdLife, Madrid, Spain, p. 95.

Mori, E., Meini, S., Strubbe, D., Ancillotto, L., Sposimo, P., Menchetti, M., 2018. Do alien free-ranging birds affect human health? A global summary of known zoonoses. In: Invasive Species and Human Health. CAB International, Wallingford UK, pp. 120–129.

Morse, S.S., Mazet, J.A., Woolhouse, M., Parrish, C.R., Carroll, D., Karesh, W.B., Daszak, P., 2012. Prediction and prevention of the next pandemic zoonosis. Lancet 380 (9857), 19561965. https://doi.org/10.1016/S0140-6736(12)61684-5.

Mühldorfer, K., 2013. Bats and bacterial pathogens: a review. Zoonoses Public Health 60 (1), 93–103. https://doi.org/10.1111/j.1863-2378.2012.01536.x.

Murton, R.K., Westwood, N.J., 1966. The foods of the rock dove and feral pigeon. Bird Study 13 (2), 130–146. https://doi.org/10.1080/00063656609476116.

Najberek, K., Olszańska, A., Tokarska-Guzik, B., Mazurska, K., Dajdok, Z., Solarz, W., 2022. Invasive alien species as reservoirs for pathogens. Ecol. Indic. 139, 108879 https://doi.org/10.1016/j.ecolind.2022.108879.

Navarro, J., Grémillet, D., Afán, I., Miranda, F., Bouten, W., Forero, M.G., Figuerola, J., 2019. Pathogen transmission risk by opportunistic gulls moving across human landscapes. Sci. Rep. 9 (1), 10659 https://doi.org/10.1038/s41598-019-46326-1.

Palmer, S.R., Soulsby, L., Simpson, D.I.H., 1998. Zoonoses: Biology, Clinical Practice, and Public Health Control. Oxford university press.

Parrott, D., Roy, S., Baker, R., Cannon, R., Eyre, D., Hill, M., Mumford, J., 2009. Horizon scanning for new invasive non-native animal species in England. In: Natural England Commissioned Report NECR009.

Perez-Sancho, M., García-Seco, T., Porrero, C., García, N., Gomez-Barrero, S., Cámara, J. M., Álvarez, J., 2020. A ten-year-surveillance program of zoonotic pathogens in feral pigeons in the City of Madrid (2005–2014): the importance of a systematic pest control. Res. J. Vet. Sci. 128, 293–298. https://doi.org/10.1016/j.rvsc.2019.12.006.

Pianka, E.R., 1973. The structure of lizard communities. Annu. Rev. Ecol. Syst. 4, 53–77. https://doi.org/10.1146/annurev.es.04.110173.000413.

Pineda-Pampliega, J., Ramiro, Y., HerreraDueñas, A., MartinezHaro, M., Hernández, J. M., Aguirre, J.I., Höfle, U., 2021. A multidisciplinary approach to the evaluation of the effects of foraging on landfills on white stork nestlings. Sci. Total Environ. 775, 145197 https://doi.org/10.1016/j.scitotenv.2021.145197.

Postigo, J.L., CarrilloOrtiz, J., Domènech, J., Tomàs, X., Arroyo, L., Senar, J.C., 2021. Dietary plasticity in an invasive species and implications for management: the case of the monk parakeet in a Mediterranean city. Anim. Biodivers. Conserv. 44.2, 185194. https://doi.org/10.32800/abc.2021.44.0185.

Prasad, A.B., Allard, M.W., NISC Comparative Sequencing Program, Green, E.D., 2008. Confirming the phylogeny of mammals by use of large comparative sequence data sets. Mol. Biol. Evol. 25 (9), 1795–1808. https://doi.org/10.1093/molbev/msn104.

Prum, R.O., Berv, J.S., Dornburg, A., Field, D.J., Townsend, J.P., Lemmon, E.M., Lemmon, A.R., 2015. A comprehensive phylogeny of birds (Aves) using targeted next-generation DNA sequencing. Nature 526 (7574), 569–573. https://doi.org/10.1038/nature15697.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41 (D1), D590–D596. https://doi.org/10.1093/nar/gks1219.

R Core Team, 2021. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL. https://www.Rproject.org/.

Rahman, M.T., Sobur, M.A., Islam, M.S., Ievy, S., Hossain, M.J., El Zowalaty, M.E., Ashour, H.M., 2020. Zoonotic diseases: etiology, impact, and control. Microorganisms 8 (9), 1405. https://doi.org/10.3390/microorganisms8091405.

Razzauti, M., Galan, M., Bernard, M., Cheval, J., Maman, S., Charbonnel, N., Cosson, J.F., 2014. Zoonotic bacterial survey assessed by next-generation sequencing. Parasit. Vectors 7 (1), 1. https://doi.org/10.1186/1756-3305-7-S1-O14.

Rodríguez-Pastor, R., Senar, J.C., Ortega, A., Faus, J., Uribe, F., Montalvo, T., 2012. Distribution patterns of invasive Monk parakeets (*Myiopsitta monachus*) in an urban habitat. Anim. Biodivers. Conserv. 35 (1), 107117.

Rohr, J.R., Barrett, C.B., Civitello, D.J., Craft, M.E., Delius, B., DeLeo, G.A., Tilman, D., 2019. Emerging human infectious diseases and the links to global food production. Nat. Sustain. 2 (6), 445–456. https://doi.org/10.1038/s41893-019-0293-3.

Rothenburger, J.L., Himsworth, C.H., Nemeth, N.M., Pearl, D.L., Jardine, C.M., 2017. Environmental factors and zoonotic pathogen ecology in urban exploiter species. EcoHealth 14, 630–641. https://doi.org/10.1007/s10393-017-1258-5.

Rulison, E.L., Luiselli, L., Burke, R.L., 2012. Relative impacts of habitat and geography on raccoon diets. Am. Midl. Nat. 168 (2), 231–246. https://doi.org/10.1674/0003-0031-168.2.231.

Shokralla, S., Spall, J.L., Gibson, J.F., Hajibabaei, M., 2012. Next-generation sequencing technologies for environ- mental DNA research. Mol. Ecol. 21, 1794–1805. https://doi.org/10.1111/j.1365-294X.2012.05538.x.

Silva, N., Igrejas, G., Gonçalves, A., Poeta, P., 2012. Commensal gut bacteria: distribution of *Enterococcus* species and prevalence of *Escherichia coli* phylogenetic groups in animals and humans in Portugal. Ann. Microbiol. 62 (2), 449–459. https://doi.org/10.1007/s13213-011-0308-4.

Slifko, T.R., Smith, H.V., Rose, J.B., 2000. Emerging parasite zoonoses associated with water and food. Int. J. Parasitol. Parasites Wildl. 30 (12–13), 1379–1393. https://doi.org/10.1016/S0020-7519(00)00128-4.

Songer, J.G., 2010. Clostridia as agents of zoonotic disease. Vet. Microbiol. 140 (3–4), 399–404. https://doi.org/10.1016/j.vetmic.2009.07.003.

Srivathsan, A., Ang, A., Vogler, A.P., Meier, R., 2016. Fecal metagenomics for the simultaneous assessment of diet, parasites, and population genetics of an understudied primate. Front. Zool. 13 (1), 113. https://doi.org/10.1186/s12983-016-0150-4.

Stensvold, C.R., Jirků-Pomajbíková, K., Tams, K.W., Jokelainen, P., Berg, R.P., Marving, E., Nielsen, H.V., 2021. Parasitic intestinal protists of zoonotic relevance detected in pigs by metabarcoding and real-time PCR. Microorganisms 9 (6), 1189. https://doi.org/10.3390/microorganisms9061189.

Stukenholtz, E.E., Hailu, T.A., Childers, S., Leatherwood, C., Evans, L., Roulain, D., Stevens, R.D., 2019. Ecology of feral pigeons: population monitoring, resource selection, and management practices. In: Wildlife Population Monitoring. IntechOpen, Dallas, TX, USA, pp. 1–15.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., Willerslev, E., 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Mol. Ecol. 21, 2045–2050. https://doi.org/10.1111/j.1365-294X.2012.05470.x.

Taylor, L.H., Latham, S.M., Woolhouse, M.E., 2001. Risk factors for human disease emergence. Philos. Trans. R. Soc. B 356 (1411), 983–989. https://doi.org/10.1098/rstb.2001.0888.

Valdez, V., Alvares, F., Layna, J.F., González, J.L., Herrera, J., de Lucas, J., Rosalino, L. M., 2022. Raccoon (*Procyon lotor*) in Iberia: status update and suitable habitats for an invasive carnivore. J. Nat. Conserv. 66, 126142 https://doi.org/10.1016/j.jnc.2022.126142.

Vogtmann, E., Chen, J., Amir, A., Shi, J., Abnet, C.C., Nelson, H., Sinha, R., 2017. Comparison of collection methods for fecal samples in microbiome studies. Am. J. Epidemiol. 185 (2), 115–123. https://doi.org/10.1093/aje/kww177.

Walsh, M.G., Sawleshwarkar, S., Hossain, S., Mor, S.M., 2020. Whence the next pandemic? The intersecting global geography of the animalhuman interface, poor health systems and air transit centrality reveals conduits for highimpact spillover. One Health 11, 100177. https://doi.org/10.1016/j.onehlt.2020.100177.

Wei, T., Simko, V., 2021. R package 'corrplot': visualization of a correlation matrix. R package version 0.92. https://github.com/taiyun/corrplot.

Whelan, C.J., Brown, J.S., Hank, A.E., 2015. Diet preference in the House Sparrow Passer domesticus: hooked on millet? Bird Study 62 (4), 569–573. https://doi.org/10.1080/00063657.2015.1089838.

Woods, R., Reiss, A., CoxWitton, K., Grillo, T., Peters, A., 2019. The importance of wildlife disease monitoring as part of global surveillance for zoonotic diseases: the role of Australia. Trop. Med. Infect. 4 (1), 29. https://doi.org/10.3390/tropicalmed4010029.

Woolhouse, M.E., Gowtage-Sequeria, S., 2005. Host range and emerging and reemerging pathogens. Emerg. Infect. Dis. 11 (12), 1842. https://doi.org/10.3201/eid1112.050997.

Ziege, M., Bierbach, D., Bischoff, S., Brandt, A.L., Brix, M., Greshake, B., Plath, M., 2016. Importance of latrine communication in European rabbits shifts along a rural–to–urban gradient. BMC Ecol. 16, 1–12. https://doi.org/10.1186/s12898-016-0083-y.