One Health 19 (2024) 100924

Contents lists available at ScienceDirect

One Health

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

One Health Farming: Noninvasive monitoring reveals links between farm vertebrate richness and pathogen markers in outdoor hoofstock

Gloria Herrero-García [a,1], Marta Pérez-Sancho [b,c,1], Patricia Barroso [a], Carmen Herranz-Benito [b], David Relimpio [d], Teresa García-Seco [b], Alberto Perelló [d], Alberto Díez-Guerrier [b], Pilar Pozo [d], Ana Balseiro [a,e], Lucas Domínguez [b,c], Christian Gortázar [d,*]

[a] Department of Animal Health, Faculty of Veterinary Medicine, Universidad de León, León 24071, Spain
[b] VISAVET Health Surveillance Center, Complutense University of Madrid, Madrid 28040, Spain
[c] Department of Animal Health, Faculty of Veterinary, Complutense University of Madrid, Madrid 28040, Spain
[d] SaBio Instituto de Investigación en Recursos Cinegéticos (IREC) CSIC-UCLM-JCCM, Ciudad Real 13071, Spain
[e] Department of Animal Health, Mountain Livestock Institute (IGM, CSIC-ULE), Finca Marzanas, Grulleros, León 24346, Spain

ARTICLE INFO

Keywords:
Farmland
Noninvasive sampling
Vertebrate community
Wildlife-livestock interface

ABSTRACT

Outdoor farming contributes to biodiversity conservation and enhances animal welfare, but also raises biosafety concerns due to livestock contact with potentially infected wildlife. Thus, there is a need to assess the balance between vertebrate species richness on farms, visits by wildlife species posing a biosafety risk, and pathogen circulation in open-air farming systems. We explored these links in a pilot study involving 15 open-air hoofstock farms (6 cattle, 5 small ruminant, and 4 pig farms), where we conducted interviews and risk point inspections and used two noninvasive tools: short-term camera trap (CT) deployment and environmental nucleic acid detection (ENAD). CTs were deployed to assess the richness of birds and mammals, as well as to determine the percentage of CTs detecting defined risk species. We also collected livestock feces and used sponges to sample surfaces for environmental DNA (eDNA), testing for nine pathogen markers. Total vertebrate richness ranged from 18 to 42 species, with waterholes significantly contributing to farm vertebrate richness, since 48.2 % of all wild vertebrates were detected at waterbodies, and 28.6 % were exclusively detected at waterholes. Pathogen markers detected at risk points correlated with those detected in livestock samples. Notably, the frequency of uidA marker detection correlated with the total number of pathogen markers detected per farm. Overall marker richness, an indicator of pathogen diversity, varied between farms, being higher in small ruminant farms compared to cattle or pig farms. At the farm level, wild vertebrate richness was negatively correlated with the richness of pathogen markers detected at risk points. Additionally, risk points with a higher probability of detecting more pathogen markers had lower vertebrate richness. Although CT-based assessments of vertebrate richness and ENAD-based pathogen marker detection are only indicators of actual biodiversity and farm health, respectively, our findings suggest that farmland vertebrate communities provide important ecosystem services and may help limit the circulation of multi-host pathogens.

1. Introduction

The wildlife-livestock-environment interface is a complex scenario with significant implications for biodiversity conservation, pathogen circulation, climate change mitigation, and rural economy [1,2]. There is a general agreement that increased heterogeneity in agricultural landscapes benefits both biodiversity and ecosystem services [3]. However, pathogen circulation can also benefit from these complex maintenance communities [4] and from increased interactions among key maintenance hosts at high-risk locations such as waterholes [5,6]. The interplay between biodiversity and pathogens can either enhance pathogen circulation (amplification effect) or reduce it (dilution effect) depending on the geographical scale and the pathogens involved, with generalist pathogens being more likely to be affected by biodiversity

* Corresponding author.
E-mail address: Christian.Gortazar@uclm.es (C. Gortázar).
[1] G. Herrero-García and M. Pérez-Sancho contributed equally as co − first authors.

https://doi.org/10.1016/j.onehlt.2024.100924
Received 21 June 2024; Received in revised form 17 October 2024; Accepted 26 October 2024
Available online 30 October 2024

changes [7]. In this context, one goal of those seeking to protect biodiversity and the health and well-being of humans and animals, is identifying synergies between animal farming and biodiversity conservation while minimizing the potential detrimental effect posed by the interactions between farmed animals and risk wildlife such as certain carnivores, ungulates, and birds [8,9].

Outdoor farming involves keeping animals in open-air environments temporarily or permanently. This practice contributes to biodiversity conservation and enhances animal welfare but also raises biosafety concerns [10]. Species such as red deer (*Cervus elaphus*), Eurasian wild boar (*Sus scrofa*), red fox (*Vulpes vulpes*), and European badger (*Meles meles*) are known to share relevant pathogens with domestic hoofstock, including the members of the *Mycobacterium tuberculosis* complex [MTC, [11]]. Synanthropic birds such as starlings, corvids, doves, and pigeons can serve as bridge hosts facilitating pathogen transfer between farms, urban areas, and natural habitats [12,13]. In turn, pathogens with a significant environmental transmission component often display in a spatially aggregated manner, at risk points [14], and multi-host pathogens thrive in host communities where wild and domesticated vertebrates, as well as the environment (e.g., soil, water) sustain their circulation [5]. Thus, there is a need to assess the balance between vertebrate species richness on farms, visits by wildlife species posing a biosafety risk, and pathogen circulation in open-air farming systems.

We explored these links in a pilot study involving 15 open-air hoofstock farms (6 cattle, 5 small ruminant, and 4 pig farms). We conducted interviews and risk point inspections [15] and used two noninvasive tools: short-term camera trap (CT) deployment and environmental nucleic acid detection (ENAD). CTs were deployed at risk points [16,17] and at random locations to: (1) assess the richness of medium and large wild vertebrates (birds and mammals) and (2) determine the percentage of CTs detecting defined risk species. We also collected livestock feces and used sponges to sample surfaces for environmental DNA (eDNA), testing for nine pathogen markers. Our goal was to implement multilevel noninvasive monitoring and investigate possible links between vertebrate richness and pathogen marker detection at both the farm and risk-point levels.

2. Material and methods

2.1. Sampling sites

The study included 15 open-air farms (cattle $n = 6$; small ruminant $n = 5$; and pig $n = 4$), distributed in five regions of mainland Spain (Madrid, cattle and small ruminant; Extremadura, pig; Castilla y León, cattle; Castilla La Mancha, small ruminant, and Murcia, pig). All farms were open-air, and fences restricted the mobility of livestock, although wildlife crossings could not be ruled out. Farms participating in this pilot study were chosen depending on the willingness of owners to participate and to represent all main hoofstock species as well as a broad geographical range. Characteristics of the studied farms are shown in Supplementary file 1, Table S1.

2.2. Farm visit protocol

In Spring 2022, an on-farm risk mitigation protocol adapted from Martínez-Guijosa *et al.* [18], was carried out on each study site to gather information on farm characteristics and husbandry practices and to characterize the livestock-wildlife-environment interface (Supplementary file 1, Fig. S1).

2.2.1. Cartography

General information on farm size, perimeter, and land uses of the farm and its surroundings was requested from each farm owner. Once in the farm, additional information on potential risk points (feeders, waterers, waterholes) and management practices that could pose a source of interspecies interactions were identified and recorded on a printed map with the help of the farmer.

2.2.2. Questionnaire and risk point visits

Attending to the risk points previously established, a structured questionnaire was carried out. The survey consisted of 80 open-ended and closed questions and collected information on livestock management, habitat configuration, feed supplementation, availability of water, wildlife sightings, and hunting activity (Supplementary file 2).

After the interview, all potential risk points were visited, georeferenced, and characterized (Supplementary file 2). Risk points were divided into watering sites (waterers and waterholes) and feeding sites (straw/hay feeders, concentrate feeders). Additionally, signs of wildlife presence (tracks, rootings, droppings, etc.) were inspected along the waterholes and randomly chosen lines (Supplementary file 1, Table S2). The frequency-based indirect index of abundance (FBII) was calculated for wild boar, and red deer according to Acevedo *et al.* [19].

2.2.3. Camera trapping

During the field visit, 16–30 (median 30) CTs (Browning Strike Force HD ProX, Browning Arms Company®, Morgan, Utah, USA) were deployed per farm. Twenty were deployed targeting previously characterized risk points and 10 were set up at random sites in the farm premises (avoiding water and food sites), as control points (Supplementary file 1, Table S3). CTs were programmed to be operative 24 h (for 2 days), to take 3 shots per motion detected (RPF-3), with one-minute time lapse between consecutive activations.

2.3. Risk species

Eight main wild vertebrate risk species (four mammals and four birds) were specifically considered according to their possible role as bridge hosts, higher abundance, and epidemiological relevance in infections shared between wildlife and livestock: wild boar, red deer, red fox, European badger, Eurasian collared dove (*Streptopelia decaocto*), spotless starling (*Sturnus unicolor*), Eurasian magpie (*Pica pica*), and rock dove (*Columba livia*) [13,20,21].

2.4. Noninvasive pathogen marker detection

On each of the 15 pilot sites, ENAD sampling was performed taking surfaces and feces samples, as follows: 20 samples (Supplementary file 1, Table S4) were taken from environmental/animal surfaces by using cellulose dry sponges (3 M Dry-Sponge; 3 M, Saint Paul, Minnesota, EEUU) prehydrated with 15 mL of an isotonic surfactant liquid that preserves genetic material while inactivating microorganisms [22]. Surface samples were obtained from risk point surfaces and the animals' hock area. In addition, ten fresh feces samples were collected (10 g/sample) in containers with 90 mL of the surfactant liquid and 3 mm diameter glass spheres to homogenize feces in the field.

The extraction and purification of DNA from surfaces and fecal samples were performed using the QIAamp Fast DNA Stool Mini Kit (Qiagen Hilden, Germany), starting from the pellet obtained after centrifuging 900 μL of the sample for 3 min at 13.000 rpm.

On-farm health surveillance focused on pathogens that targeted the 3 livestock groups (cattle, small ruminant, and pig) and were shared with wildlife species. In each sample, nine bacterial pathogen markers were evaluated by specific real-time PCR protocols previously described (Table 1). Any eDNA signal was considered positive.

Results of the annual compulsory tuberculosis skin testing for the 6 participating cattle farms were provided by the Regional Veterinary Authorities [23]. Data were obtained through the official single intradermal tuberculin test.

2.5. Statistical analysis

Initial data exploration was performed to check for variable

Table 1
Shared pathogen markers studied in surface samples and feces.

Pathogen marker	Detection/ Characterization*	Real time PCR protocol
IS*6110*	MTC	Lorente-Leal *et al.* [24]
IS*900*	MAP	Kim *et al.* [25]
IS*711*	*Brucella* spp.	Bounaadja *et al.* [26]
IS*1111*	*Coxiella burnetii*	Klee *et al.* [27]
invA	*Salmonella enterica*	Hoorfar *et al.* [28]
uidA	*Escherichia coli*	Cabal *et al.* [29]; Cabal *et al.* [30]
*stx*1	Shiga toxin-producing *E. coli*	Foodproof STEC Screening Lyokit, Biotecon diagnosis GmbH, Postdam, Germany
*stx*2	Shiga toxin-producing *E. coli*	
eae	Shiga toxin-producing *E. coli*	

MTC: *Mycobacterium tuberculosis* complex; MAP: *M. avium subspecies paratuberculosis*; *First 6 pathogen markers were used for pathogen detection; last 3 pathogen markers were used for characterization of Shiga toxin-producing *Escherichia coli*.

parametric assumptions and transformation needs. Non-parametric tests for non-normally distributed explanatory variables were performed to assess differences between livestock production systems, risk points, and sample types. These relationships were assessed using Kruskal-Wallis tests for continuous data and Chi-square tests for categorical data. Differences among groups were explored through multiple comparison post-hoc analyses, with Bonferroni corrections. To check for linear relationships, Spearman's rank correlation tests were used.

A cumulative link mixed model (CLMM) for ordinal regression with logit as the link function, was fit to evaluate the relationship between pathogen richness (0 / 1 / 2 or more) in each sponge sample (taken at environmental-risk point surfaces) and a set of potential farm management and vertebrate related variables (Supplementary file 1, Table S5). Statistically significant variables at the level of 0.25 (univariable analyses) were then used as covariates for stepwise regression. The farm was included as a random factor. Likelihood ratio tests and corrected Akaike Information Criterion (AICc) were used to establish whether the inclusion of main effects and interaction effects significantly improved the model. The CLMM was fitted in the library "*ordinal*" [31]. Tukey's tests were used for post-hoc comparisons among means.

Collinearity among explanatory variables was explored by Variance Inflation Factor (VIF) analysis [32]. Once the best model was selected, goodness-of-fit and the absence of residual patterns in data variation were checked. Significance was set at $p < 0.05$. Results in the model were expressed as odds ratios (ORs) with 95 % confidence intervals (CIs). All statistical analyses were conducted using the computing R software 4.3.2.

3. Results

3.1. Wild vertebrates in open-air farms

3.1.1. Species richness

On-farm vertebrate species richness, as recorded by CTs, was large as evidenced by the number of wild vertebrate species recorded per farm [range 16–37, median 20; Supplementary file 3, Fig. S1]. Wild mammal richness ranged from 2 to 9 (median 7) and total vertebrate richness ranged from 18 to 42 (median 24) (Supplementary file 3, Table S1). Differences between farmed species were not significant ($p > 0.05$, Chi-square test).

We found differences between risk point types in the percentage of CTs detecting wild mammals and birds (Supplementary file 3, Fig. S2). Wild vertebrate species richness recorded at waterbodies ranged from 9 to 31 (median 13); at feeders from 1 to 11 (median 8); and at random points from 6 to 19 [median 10, $p < 0.001$, Kruskal Wallis test, (Fig. 1)]. Waterholes and other waterbodies contributed remarkably to farm vertebrate richness, since 48.2 % of all wild vertebrates detected on farms were detected at waterbodies, and 28.6 % were exclusively detected at waterholes (Fig. 1).

3.1.2. Risk species

The most often detected species at risk points was the red fox (detected on 32 % of the cameras, $n = 81/244$), followed by the wild boar (17.6 %, $n = 43/244$). Wild boar and badgers were more frequently detected at cattle farm risk points (Supplementary file 3, Fig. S3), compared to small ruminant or pig farm risk points ($p = 0.005$, $p = 0.002$, Chi-square test, respectively). The most often detected bird at risk points was the spotless starling (16 %, $n = 39/244$). The four main bird species were mostly detected at pig farm risk points, rather than in small ruminant or cattle farms [Chi-square test, $p < 0.001$, $p = 0.008$, $p = 0.001$, $p < 0.01$, respectively, (Supplementary file 3, Fig. S3)].

Cameras set up at random points (Supplementary file 3, Fig. S4) detected most frequently the red fox (25.3 %, $n = 44/174$), followed by the wild boar (21.8 %, $n = 38/174$). The four wild mammal risk species were mostly detected in cattle farm random points, rather than in small ruminant or pig farms, although no statistical differences were found ($p > 0.05$, Chi-square test). The spotless starling was the most frequently detected risk bird at cameras set up at random points [(Supplementary file 3, Fig. S4), 7.5 %, $n = 13/174$].

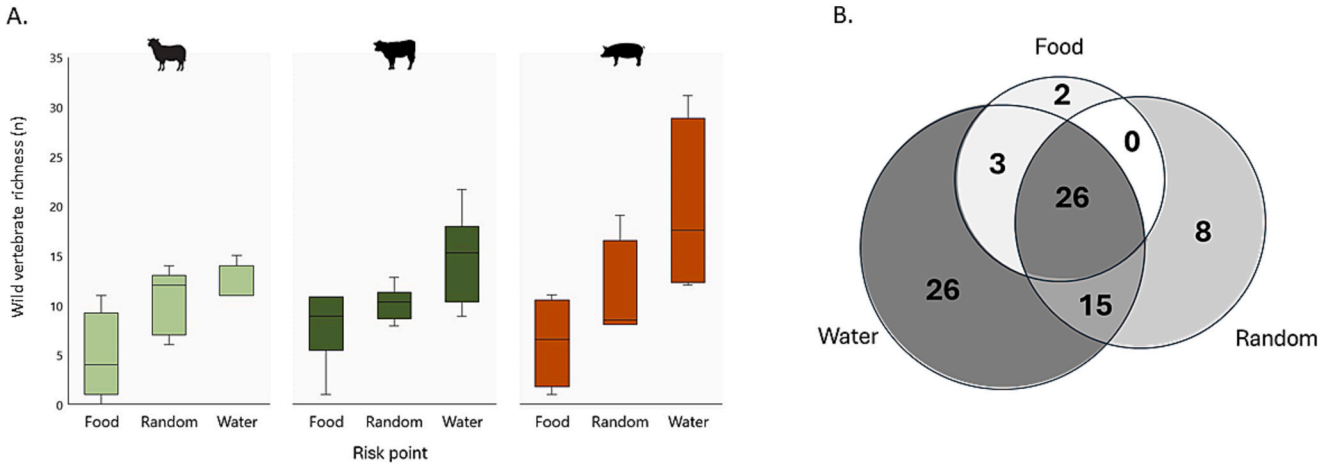

Fig. 1. Wild vertebrate richness recorded at 15 open-air farms, per risk point (water, food, random). (A) Differences between farmed species are shown. From left to right, small ruminant ($n = 5$), cattle ($n = 6$), and pig ($n = 4$) farms. Boxplots show interquartile range, minimum and maximum data; horizontal lines in the box represent medians. (B) Number of species detected at each risk point. Darker colors indicate higher number of species recorded by camera traps.

3.2. Pathogen markers in the farm environment

Pathogen markers detected at risk points were correlated with pathogen markers detected in samples from livestock (sponges taken on the animal + feces) ($r_s = 0.69$, $p = 0.005$, Table 2). Regarding detection probability, detecting the IS*6110* gene was 3 times more likely in sponges than in feces, with no difference between sponges taken on the animal, at waterers, or on feeders. Detecting the IS*900* gene was 5 times more likely in sponges than in feces. IS*900* gene detection occurred more often in animal sponges than in environmental ones. IS*1111* was only detected in sponges, never in feces. *uidA* and *invA* genes were as frequently detected in feces as in all kinds of sponges. By contrast, the virulence markers *Stx*1, *Stx*2, and *eae* were more often detected in feces than in sponges (5, 4, and 2 times more, respectively). The IS*711* gene was only detected on one occasion. The frequency of *uidA* marker detection correlated with the total number of pathogen markers detected per farm in sponge samples collected at water or food risk points ($r_s = 0.74$, $p < 0.05$).

By farmed animal species, all six pathogen markers were detected in small ruminant farms, while only three (IS*6110*, *invA*, and *uidA*) were also detected in cattle and pig farms (Fig. 2). In small ruminant farms, we detected the IS*6110* gene in 33.7 % of the sponge samples and in all five farms visited. In one small ruminant farm, and after positive (IS*6110* gene) noninvasive sampling, MTC infection was confirmed by direct PCR and immunohistochemistry in lung and lymph node tissues from two slaughtered adult sheep (Supplementary file 3, Fig. S5). Five of the 6 cattle farms tested tuberculosis positive in the official single intradermal tuberculin testing in 2022. Three of these were further positive to PCR and culture. The only cattle farm that tested negative for the skin test also resulted negative for the IS*6110* gene in animal sponges and feces.

The overall marker richness (number of pathogen and virulence markers detected in feces and sponges), an indicator of pathogen diversity, varied between farms (range 2–8, median 6), being higher in small ruminant (median 7 range 6–8) than in cattle (median 6, range 4–6) or pig farms (median 4; range 2–5, $p = 0.036$, Chi-square test).

Table 2
Results of nine genetic markers for environmental nucleic acid detection (ENAD), by sample type.

Marker	Number of positive samples/sample size (% positive samples)				
	Feces	Sponges			
		Animal	Water	Food	Total
IS*6110*	11/145 (7.59)	31/149 (20.81)	12/79 (15.20)	13/62 (20.97)	67/290 (23.10)
IS*900*	2/145 (1.38)	15/149 (10.07)	2/79 (2.53)	3/62 (4.84)	20/290 (6.87)
IS*1111*	0/145 (0.00)	13/149 (8.72)	3/79 (3.80)	3/62 (4.84)	19/290 (6.55)
IS*711*	0/145 (0.00)	0/149 (0.00)	1/79 (1.27)	0/62 (0.00)	1/290 (0.34)
uidA	125/145 (86.21)	133/149 (89.26)	68/79 (86.08)	47/62 (75.81)	248/290 (85.52)
invA	13/145 (8.97)	10/149 (6.71)	12/79 (15.19)	5/62 (8.06)	27/290 (9.31)
*Stx*1	47/145 (32.41)	16/149 (10.74)	1/79 (1.27)	2/62 (3.23)	19/290 (6.55)
*Stx*2	36/145 (24.83)	14/149 (9.40)	2/79 (2.53)	2/62 (3.23)	18/290 (6.21)
eae	20/145 (13.79)	18/149 (12.08)	1/79 (1.27)	3/62 (4.84)	22/290 (7.59)

IS*6110*: *Mycobacterium tuberculosis* complex; IS*900*: *M. avium subspecies paratuberculosis*; IS*1111*: *Coxiella burnetii*; IS*711*: *Brucella* spp.; *invA*: *Salmonella enterica*; *uidA*: *Escherichia coli*; *Stx*1, *Stx*2 and *eae*: Shiga toxin-producing *E. coli*; animal sponges: sampled on the hock's area; water sponges: sampled on troughs, waterholes, or other water sites; food sponges: sampled on feeders, hay bundles or other food sites.

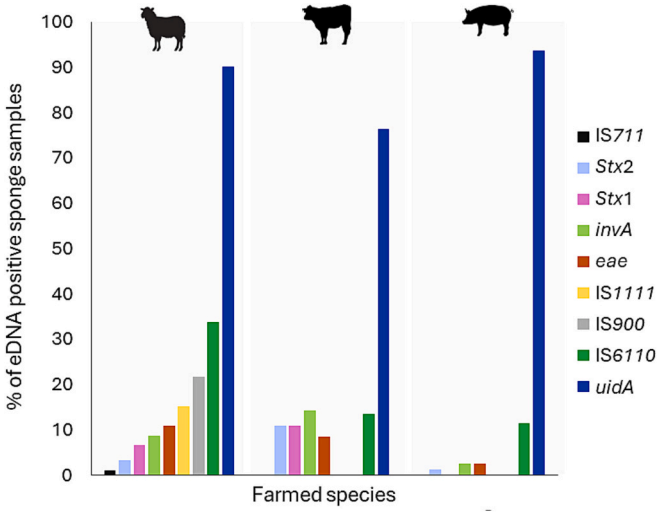

Fig. 2. Percentage of environmental DNA positive sponge samples by farmed species. Black silhouettes indicate livestock production type (from left to right, small ruminant, cattle, and pig). IS*711*: *Brucella* spp.; *Stx*1, *Stx*2 and *eae:* Shiga toxin-producing *E. coli; invA*: *Salmonella enterica*; IS*6110*: *Mycobacterium tuberculosis* complex; IS*1111*: *Coxiella burnetii;* IS*900*: *M. avium subspecies paratuberculosis*; *uidA*: *Escherichia coli.*

3.3. Links between pathogen markers and vertebrate richness

At the farm level, wild vertebrate richness and the richness of pathogen markers detected on environmental sponges taken at risk points were negatively correlated [$r_s = -0.65$, $p = 0.008$, Fig. 3, (Supplementary file 3, Tables S2 and S3)].

Results of the CLMM model revealed that risk points with a higher probability of detecting more pathogen markers had lower vertebrate richness (≤ 2, OR 0.24, 95 % CI = 0.08–0.74), belonged to small ruminant farms (OR 4.78, 95 % CI = 1.52–15.01), and had a lower number of waterholes per plot [OR 0.55, 95 % CI = 0.30–0.99, Table 3, (Supplementary file 3, Fig. S6)].

4. Discussion

Combining two noninvasive tools, CTs and ENAD, we assessed vertebrate richness and detected pathogen markers on small ruminant,

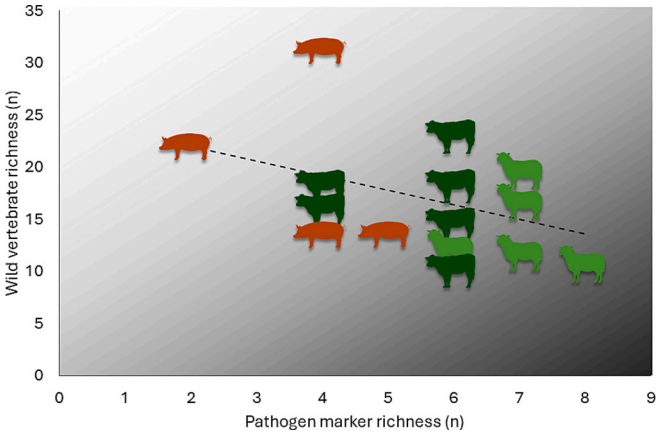

Fig. 3. Overall marker richness against wild vertebrate richness. The dotted line shows the linear regression. Richness is calculated by the number of species detected at risk points. Silhouettes and colors indicate farmed species (light green for small ruminant farms, dark green for cattle farms, and orange for pig farms). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Results of the cumulative link mixed model (CLMM) at the risk point level. Dependent variable used: No pathogen markers/ One pathogen marker/ Two or more pathogen markers. The farm was included as a random term in the model. Vertebrate richness is categorized as: low (≤2 vertebrate species detected), high (>2- ≤ 5), very high (>5).

	Dependent variable: Pathogen markers		
Predictors	*Odds Ratio*	*95 % CI*	*p*
0I1	0.05	0.02–0.18	**<0.001**
1I2	3.21	1.12–9.14	**0.029**
Vertebrate richness (Low)	–	–	
Vertebrate richness (High)	0.24	0.08–0.74	**0.012**
Vertebrate richness (Very high)	1.20	0.35–4.13	0.777
Number of waterholes per plots	0.55	0.30–0.99	**0.047**
Livestock (Cattle)	–	–	
Livestock (Pig)	1.65	0.48–5.73	0.430
Livestock (Small ruminant)	4.78	1.52–15.01	**0.007**

CI: Confidence Interval. In bold significant values.

cattle, and pig open-air farms. Our results revealed a negative correlation between wild vertebrate richness and pathogen marker detection at the farm level. Additionally, risk points with higher pathogen marker detection showed lower vertebrate richness.

Waterholes and other lentic waterbodies were found to be particularly relevant for farm vertebrate richness, as seen elsewhere [33]. This is relevant considering that waterhole characteristics also modulate the likelihood of pathogen maintenance and transmission. These results suggest that targeted interventions, e.g., adjusting the number and placement of waterholes and regulating livestock access, might benefit both biodiversity conservation and disease control efforts [6].

However, farm biodiversity also comes along with the presence of species that pose risk. Previous studies have shown that the red fox carries a wide range of pathogens shared with both, domestic animals and humans [34]. Wild boar and badgers were most frequently detected on cattle farms, which raises concern because they are considered part of the MTC maintenance host community and may hinder bovine TB control efforts [35,36]. Wild boar were also detected on 2 out of 4 open-air pig farms, underscoring the need for improved biosafety measures in light of the emerging threat of African Swine Fever in Europe [37]. Additionally, common farmland birds such as the spotless starling were more frequently observed at pig farm risk points, which is concerning due to their potential role as bridge hosts for pathogens and antimicrobial resistance genes between different habitats [13].

Environmental nucleic acid detection emerges as a useful tool for addressing pathogen surveillance challenges posed by complex maintenance communities across diverse epidemiological scenarios [38]. Significant progress has been made in the debate on result interpretation, specifically regarding nucleic acid detection vs. pathogen viability, at least for certain pathogens [39]. ENAD offers several advantages, including avoiding direct host sampling, thereby minimizing stress for hosts and reducing both the sampling costs and effort.

In terms of pathogen and virulence marker detection, we observed that the markers IS*6110*, IS*900*, IS*711*, IS*1111*, *invA*, and *uidA* were at least as detectable in sponge samples as in feces, while *Stx*1, *Stx*2, and *eae* were more frequently detected in fecal samples. This is a significant finding as it suggests a convenient noninvasive sampling method for detecting multiple pathogen markers in extensive farm environments, similar to previous findings for MTC [38]. Unlike fecal samples, sponge samples collect genetic material that could stem from a broader portion of the herd, whereas feces reflect individual contributions. Additionally, sponges are temperature-resistant and biosafe, as pathogens are fixed in preserving liquid [22]. The frequency of *E. coli* marker detection correlated with the total number of pathogen markers detected per farm, suggesting that the level of environmental *E. coli* contamination could serve as an indicator of pathogen richness. Another relevant finding was that small ruminant farms exhibited the highest diversity of pathogen markers. Markers IS*900*, IS*711*, and IS*1111* were exclusively detected on small ruminant farms, and IS*6110* was detected on all five farms. This underscores the importance of small ruminants in TB epidemiology [40] and suggests that they warrant increased veterinary surveillance.

Richer farmland vertebrate communities were associated with lower pathogen richness, a finding that aligns with observations in natural habitats [41]. However, this overall trend may not apply to all specific host-pathogen groups, as it can vary depending on host community composition and environmental factors [5].

Camera trapping methods and ENAD specificity were among the limitations of this study. Measuring biodiversity is complex and requires attention to a wide range of organisms and scales, not just on warm-blooded vertebrates [42]. Additionally, CTs capture only a portion of the total vertebrate richness [43], and a 48-h sampling period may be insufficient to completely assess all detectable species, leading to potentially underestimated richness [44]. However, the consistent effort applied across all farms allowed controlling this bias. Moreover, the short setup period was compatible with fieldwork logistics and yielded valuable data for vertebrate richness and risk assessment purposes, including the detection of all eight relevant risk species.

Regarding ENAD, nucleic acid detection, especially at lower DNA signals, does not necessarily indicate the presence of viable organisms at sufficient concentration to be infective (but see [39]), and we could be overestimating the results since any eDNA signal was considered positive. Additionally, finding pathogen eDNA in sponge samples, even in those collected from animals, does not confirm its presence in the farmed species but rather in the broader farm ecosystem, including other farmed species, wildlife, and the environment [38]. A controversy exists over the association between eDNA signal and pathogen presence (revised by [45]). In this context, it is important to emphasize that we used the eDNA signals to study the relationship between vertebrate richness and the presence of microorganisms of interest, not for diagnostic purposes. Nonetheless, our findings are valuable for surveillance and monitoring. For instance, the low-specific marker IS*6110* for MTC showed positive results in the farm environments of 5 out of 6 cattle herds, all of which tested positive during the skin testing rounds in 2022. We also detected MTC marker positivity in small ruminant farms and confirmed MTC infection at slaughter, supporting the potential utility of sponges in TB eradication programs. In the small ruminant farms, MAP and *Coxiella burnetii* infection had been previously confirmed [46,47].

5. Conclusion

The relatively small number of study farms limits our ability to infer associations. However, our findings suggest that sustainably managed open-air farming systems may contribute to biodiversity conservation by maintaining vital habitats, including lentic waterbodies. While diverse host communities inevitably include risk species that pose a threat of infection entry or maintenance, we found that varied farmland vertebrate communities, which provide ecosystem services, may help limit the circulation of multi-host pathogens. Although CT-based assessments of vertebrate richness and ENAD-based pathogen marker detection are only indicators of actual biodiversity and farm health, we postulate that combining both approaches can yield valuable insights for conservation planning, particularly regarding which production systems, species, or risk areas require enhanced prevention efforts. We anticipate that this combination will facilitate farm biosafety monitoring and improve the effectiveness of future interventions.

Funding

This work was supported by the Spanish MCIN/AEI/10.13039/501100011033/ and the European Union NextGeneration EU/PRTR [grant number PLEC2021-008113]; and the MCIN/AEI/10.13039/501100011033/FEDER, UE [grant numbers PID2022-141906OB-C21, PID2022-141906OB-C22]. GHG was funded by Junta de Castilla y León

and FSE [grant number LE036-20]. PB by Juan de la Cierva Formación 2021, NextGenerationEU, PRTR [grant number DC2022–049103-I]. A.P by Universidad de Castilla-La Mancha and FSE [grant number 2023-PRED-36011]. PP by Juan de la Cierva Formación 2021, funded by the Ministerio de Ciencia e Innovación, Agencia Estatal de Investigación and NextGenerationEU/PRTR [grant number FJC2021–046805-I].

Ethics statement

The University of Castilla-La Mancha (UCLM) research ethics committee granted a formal waiver of ethics approval, since only routine veterinary care was involved in this study. In addition, Ethics approval was unnecessary according to Spanish national regulations (Real Decreto 53/2013).

CRediT authorship contribution statement

Gloria Herrero-García: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Marta Pérez-Sancho:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Conceptualization. **Patricia Barroso:** Writing – review & editing, Visualization, Validation, Investigation, Formal analysis, Data curation. **Carmen Herranz-Benito:** Writing – review & editing, Validation, Investigation, Data curation. **David Relimpio:** Writing – review & editing, Validation, Investigation, Data curation. **Teresa García-Seco:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Conceptualization. **Alberto Perelló:** Writing – review & editing, Validation, Investigation, Data curation. **Alberto Díez-Guerrier:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Data curation. **Pilar Pozo:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. **Ana Balseiro:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Lucas Domínguez:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Christian Gortázar:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank our colleagues from LABOCOR, MAEVA, SaBio-IREC, VISAVET and the University of León for their help and support. Special thanks to the farmers, farm owners and veterinaries, thanks to whom this work could not have been carried out.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2024.100924.

Data availability

Data will be made available on request.

References

[1] S. Morand, Emerging diseases, livestock expansion and biodiversity loss are positively related at global scale, Biol. Conserv. 248 (2020) 1–9, https://doi.org/10.1016/j.biocon.2020.108707.
[2] L. Thompson, J. Rowntree, W. Windisch, S.M. Waters, L. Shalloo, P. Manzano, Ecosystem management using livestock: embracing diversity and respecting ecological principles, Anim. Front. 13 (2023) 28–34, https://doi.org/10.1093/af/vfac094.
[3] S. Petit, D.A. Landis, Landscape-scale management for biodiversity and ecosystem services, Agric. Ecosyst. Environ. 347 (2023), https://doi.org/10.1016/j.agee.2023.108370.
[4] A. Frainer, B.G. McKie, P.A. Amundsen, R. Knudsen, K.D. Lafferty, Parasitism and the biodiversity-functioning relationship, Trends Ecol. Evol. 33 (2018) 260–268, https://doi.org/10.1016/j.tree.2018.01.011.
[5] P. Barroso, D. Relimpio, J.A. Zearra, J.J. Cerón, P. Palencia, B. Cardoso, E. Ferreras, M. Escobar, G. Cáceres, J.R. López-Olvera, C. Gortázar, Using integrated wildlife monitoring to prevent future pandemics through one health approach, One Health 16 (2023), https://doi.org/10.1016/j.onehlt.2022.100479.
[6] G. Herrero-García, P. Barroso, L. Preite, D. Relimpio, R. Vaz-Rodrigues, A. Balseiro, C. Gortázar, Waterhole characteristics in tuberculosis positive and negative beef cattle farms from endemic regions in Spain, Rangel. Ecol. Manage. 92 (2024) 50–58, https://doi.org/10.1016/j.rama.2023.09.008.
[7] J.R. Rohr, D.J. Civitello, F.W. Halliday, P.J. Hudson, K.D. Lafferty, C.L. Wood, E. A. Mordecai, Towards common ground in the biodiversity–disease debate, Nat Ecol Evol 4 (2020) 24–33, https://doi.org/10.1038/s41559-019-1060-6.
[8] R. Sabatier, D. Durant, L. Hazard, A. Lauvie, E. Lecrivain, D. Magda, G. Martel, B. Roche, C. De Sainte Marie, F. Teillard, M. Tichit, Towards biodiversity-based livestock systems: review of evidence and options for improvement, CAB Reviews 10 (2015), https://doi.org/10.1079/PAVSNNR201510025.
[9] A.K. Wiethoelter, D. Beltrán-Alcrudo, R. Kock, S.M. Mor, Global trends in infectious diseases at the wildlife-livestock interface, PNAS 112 (2015) 9662–9667, https://doi.org/10.1073/pnas.1422741112.
[10] EFSA AHAW Panel, S.S. Nielsen, J. Alvarez, D.J. Bicout, P. Calistri, E. Canali, J. A. Drewe, B. Garin-Bastuji, J.L. Gonzales Rojas, M. Herskin, M.Á. Miranda Chueca, V. Michel, B. Padalino, P. Pasquali, H.C. Roberts, L.H. Sihvonen, H. Spoolder, K. Stahl, A. Velarde, A. Viltrop, C. Winckler, S. Blome, S. More, A. Gervelmeyer, S. Antoniou, C. Gortázar Schmidt, African swine fever and outdoor farming of pigs, EFSA J. 19 (2021), https://doi.org/10.2903/j.efsa.2021.6639.
[11] C. Gortázar, R.J. Delahay, R.A. Mcdonald, M. Boadella, G.J. Wilson, D. Gavier-Widen, P. Acevedo, The status of tuberculosis in European wild mammals, Mamm Rev 42 (2012) 193–206, https://doi.org/10.1111/j.1365-2907.2011.00191.x.
[12] C. López-Calderón, V. Martín-Vélez, J. Blas, U. Höfle, M.I. Sánchez, A. Flack, W. Fiedler, M. Wikelski, A.J. Green, White stork movements reveal the ecological connectivity between landfills and different habitats, Mov Ecol 11 (2023), https://doi.org/10.1186/s40462-023-00380-7.
[13] A. Sánchez-cano, C. López-Calderón, T. Cardona-cabrera, A.J. Green, U. Höfle, Connectivity at the human-wildlife interface : starling movements relate to carriage of *E. coli*, Sci. Total Environ. 926 (2024) 171899, https://doi.org/10.1016/j.scitotenv.2024.171899.
[14] J.K. Blackburn, H.H. Ganz, J.M. Ponciano, W.C. Turner, S.J. Ryan, P. Kamath, C. Cizauskas, K. Kausrud, R.D. Holt, N.C. Stenseth, W.M. Getz, Modeling R0 for pathogens with environmental transmission: animal movements, pathogen populations, and local infectious zones, Int. J. Environ. Res. Public Health 16 (2019), https://doi.org/10.3390/ijerph16060954.
[15] J. Martínez-Guijosa, J.F. Lima-Barbero, P. Acevedo, D. Cano-Terriza, S. Jiménez-Ruiz, J.Á. Barasona, M. Boadella, I. García-Bocanegra, C. Gortázar, J. Vicente, Description and implementation of an on-farm wildlife risk mitigation protocol at the wildlife-livestock interface: tuberculosis in Mediterranean environments, Prev. Vet. Med. 191 (2021) 1–10, https://doi.org/10.1016/j.prevetmed.2021.105346.
[16] E. Kukielka, J.A. Barasona, C.E. Cowie, J.A. Drewe, C. Gortazar, I. Cotarelo, J. Vicente, Spatial and temporal interactions between livestock and wildlife in south Central Spain assessed by camera traps, Prev. Vet. Med. 112 (2013) 213–221, https://doi.org/10.1016/j.prevetmed.2013.08.008.
[17] J.A. Barasona, J. Vicente, I. Díez-Delgado, J. Aznar, C. Gortázar, M.J. Torres, Environmental presence of mycobacterium tuberculosis complex in aggregation points at the wildlife/livestock Interface, Transbound. Emerg. Dis. 64 (2017) 1148–1158, https://doi.org/10.1111/tbed.12480.
[18] J. Martínez-Guijosa, J.F. Lima-Barbero, P. Acevedo, D. Cano-Terriza, S. Jiménez-Ruiz, J.Á. Barasona, M. Boadella, I. García-Bocanegra, C. Gortázar, J. Vicente, Description and implementation of an on-farm wildlife risk mitigation protocol at the wildlife-livestock interface: tuberculosis in Mediterranean environments, Prev. Vet. Med. 191 (2021), https://doi.org/10.1016/j.prevetmed.2021.105346.
[19] P. Acevedo, J. Vicente, U. Höfle, J. Cassinello, F. Ruiz-Fons, C. Gortazar, Estimation of European wild boar relative abundance and aggregation: a novel method in epidemiological risk assessment, Epidemiol. Infect. 135 (2007) 519–527, https://doi.org/10.1017/S0950268806007059.
[20] F. Ruiz-Fons, A review of the current status of relevant zoonotic pathogens in wild swine (Sus scrofa) populations: changes modulating the risk of transmission to humans, Transbound. Emerg. Dis. 64 (2017) 68–88, https://doi.org/10.1111/tbed.12369.
[21] J. Vicente, K.C. Vercauteren, C. Gortázar, Diseases at the Wildlife-Livestock Interface, Springer Nature, Switzerland, 2021. http://www.springer.com/series/8826.
[22] S. Barroso-Arévalo, M. Díaz-Frutos, A. Kosowska, M. Pérez-Sancho, L. Domínguez, J.M. Sánchez-Vizcaíno, A useful tool for the safe diagnosis and control of the two

main pandemics of the XXI century: COVID-19 and African swine fever disease, PloS One 18 (2023), https://doi.org/10.1371/journal.pone.0282632.
[23] PNETB, Programa Nacional de Erradicación de Tuberculosis Bovina, "Dirección general de sanidad de la producción agraria". https://www.mapa.gob.es/va/ganaderia/temas/sanidad-animal-higiene-ganadera/programatb2022_tcm39-583922.pdf, 2022 accessed December 23, 2023.
[24] V. Lorente-Leal, E. Liandris, M. Pacciarini, A. Botelho, K. Kenny, B. Loyo, R. Fernández, J. Bezos, L. Domínguez, L. De Juan, B. Romero, Direct PCR on tissue samples to detect mycobacterium tuberculosis complex: an alternative to the bacteriological culture, J. Clin. Microbiol. 59 (2021), https://doi.org/10.1128/JCM.01404-20.
[25] S.G. Kim, S.J. Shin, R.H. Jacobson, L.J. Miller, P.R. Harpending, S.M. Stehman, C. A. Rossiter, D.A. Lein, Development and application of quantitative polymerase chain reaction assay based on the ABI 7700 system (TaqMan) for detection and quantification of Mycobacterium avium subsp. paratuberculosis, J. Vet. Diagn. Invest. 14 (2002) 126–131, https://doi.org/10.1177/104063870201400206.
[26] L. Bounaadja, D. Albert, B. Chénais, S. Hénault, M.S. Zygmunt, S. Poliak, B. Garin-Bastuji, Real-time PCR for identification of Brucella spp.: a comparative study of IS711, bcsp31 and per target genes, Vet. Microbiol. 137 (2009) 156–164, https://doi.org/10.1016/j.vetmic.2008.12.023.
[27] S.R. Klee, J. Tyczka, H. Ellerbrok, T. Franz, S. Linke, G. Baljer, B. Appel, Highly sensitive real-time PCR for specific detection and quantification of Coxiella burnetii, BMC Microbiol. 6 (2006), https://doi.org/10.1186/1471-2180-6-2.
[28] J. Hoorfar, P. Ahrens, A.P. Rådstrom, R. Rådstrom, Automated 5 nuclease PCR assay for identification of salmonella enterica, J. Clin. Microbiol. 38 (2000) 3429–3435, https://doi.org/10.1128/JCM.38.9.3429-3435.2000.
[29] A. Cabal, S. Gómez-Barrero, C. Porrero, C. Bárcena, G. López, R. Cantón, C. Gortázar, L. Domínguez, J. Álvarez, Assessment of virulence factors characteristic of human Escherichia coli pathotypes and antimicrobial resistance in O157:H7 and non-O157:H7 isolates from livestock in Spain, Appl. Environ. Microbiol. 79 (2013) 4170–4172, https://doi.org/10.1128/AEM.00537-13.
[30] A. Cabal, L. Geue, S. Gómez-Barrero, S. Barth, C. Bárcena, K. Hamm, M.C. Porrero, A. Valverde, R. Cantón, C. Menge, C. Gortázar, L. Domínguez, J. Álvarez, Detection of virulence-associated genes characteristic of intestinal Escherichia coli pathotypes, including the enterohemorrhagic/enteroaggregative O104: H4, in bovines from Germany and Spain, Microbiol. Immunol. 59 (2015) 433–442, https://doi.org/10.1111/1348-0421.12275.
[31] R.H.B. Christensen, Package "Ordinal": Regression Models for Ordinal Data. https://cran.r-project.org/web/packages/ordinal/ordinal.pdf, 2023.
[32] C.G. Thompson, R.S. Kim, A.M. Aloe, B.J. Becker, Extracting the variance in flation factor and other multicollinearity diagnostics from typical regression results, Basic Appl Soc Psych 39 (2017) 81–90, https://doi.org/10.1080/01973533.2016.1277529.
[33] G. Thiere, S. Milenkovski, P.E. Lindgren, G. Sahlén, O. Berglund, S.E.B. Weisner, Wetland creation in agricultural landscapes: biodiversity benefits on local and regional scales, Biol. Conserv. 142 (2009) 964–973, https://doi.org/10.1016/j.biocon.2009.01.006.
[34] L. Lledó, J.L. Serrano, C. Giménez-Pardo, I. Gegúndez, Wild red foxes (Vulpes vulpes) as sentinels of rodent-borne hantavirus and lymphocytic choriomeningitis virus in the province of Soria, northern Spain, J. Wildl. Dis. 56 (2020) 658–661, https://doi.org/10.7589/2019-09-239.
[35] V. Naranjo, C. Gortazar, J. Vicente, J. de la Fuente, Evidence of the role of European wild boar as a reservoir of *Mycobacterium tuberculosis* complex, Vet. Microbiol. 127 (2008) 1–9, https://doi.org/10.1016/j.vetmic.2007.10.002.
[36] C. Blanco Vázquez, T.D. Barral, B. Romero, M. Queipo, I. Merediz, P. Quirós, J.Á. Armenteros, R. Juste, L. Domínguez, M. Domínguez, R. Casais, A. Balseiro, Spatial and temporal distribution of mycobacterium tuberculosis complex infection in eurasian badger (Meles meles) and cattle in Asturias, Spain, Animals 11 (2021), https://doi.org/10.3390/ani11051294.
[37] S.A. Bacigalupo, L.K. Dixon, S. Gubbins, A.J. Kucharski, J.A. Drewe, Wild boar visits to commercial pig farms in Southwest England: implications for disease transmission, Eur. J. Wildl. Res. 68 (2022), https://doi.org/10.1007/s10344-022-01618-2.
[38] J. Martínez-Guijosa, B. Romero, J.A. Infantes-Lorenzo, E. Díez, M. Boadella, A. Balseiro, M. Veiga, D. Navarro, I. Moreno, J. Ferreres, M. Domínguez, C. Fernández, L. Domínguez, C. Gortázar, Environmental DNA: a promising factor for tuberculosis risk assessment in multi-host settings, PloS One 15 (2020), https://doi.org/10.1371/journal.pone.0233837.
[39] A.C. Pereira, D. Pinto, M.V. Cunha, Unlocking environmental contamination of animal tuberculosis hotspots with viable mycobacteria at the intersection of flow cytometry, PCR, and ecological modelling, Sci. Total Environ. 891 (2023), https://doi.org/10.1016/j.scitotenv.2023.164366.
[40] M. Muñoz-Mendoza, B. Romero, A. del Cerro, C. Gortázar, J.F. García-Marín, S. Menéndez, J. Mourelo, L. de Juan, J.L. Sáez, R.J. Delahay, A. Balseiro, Sheep as a potential source of bovine TB: epidemiology, pathology and evaluation of diagnostic techniques, Transbound. Emerg. Dis. 63 (2016) 635–646, https://doi.org/10.1111/tbed.12325.
[41] M.J. Pongsiri, J. Roman, V.O. Ezenwa, T.L. Goldberg, H.S. Koren, S.C. Newbold, R. S. Ostfeld, S.K. Pattanayak, D.J. Salkeld, Biodiversity loss affects global disease ecology, Bioscience 59 (2009) 945–954, https://doi.org/10.1525/bio.2009.59.11.6.
[42] C.E. Moreno, J.M. Calderón-Patrón, V. Arroyo-Rodríguez, F. Barragán, F. Escobar, Y. Gómez-Ortiz, N. Martín-Regalado, A.P. Martínez-Falcón, M.Á. Martínez-Morales, E. Mendoza, I.J. Ortega-Martínez, C.X. Pérez-Hernández, E. Pineda, R. Pineda-López, C.L. Rios-Díaz, P. Rodríguez, F. Rosas, J.E. Schondube, I. Zuria, Measuring biodiversity in the Anthropocene: a simple guide to helpful methods, Biodivers. Conserv. 26 (2017) 2993–2998, https://doi.org/10.1007/s10531-017-1401-1.
[43] L.N. Rich, D.A.W. Miller, H.S. Robinson, J.W. McNutt, M.J. Kelly, Using camera trapping and hierarchical occupancy modelling to evaluate the spatial ecology of an African mammal community, J. Appl. Ecol. 53 (2016) 1225–1235, https://doi.org/10.1111/1365-2664.12650.
[44] P. Barroso, J.R. López-Olvera, T. Kiluba Wa Kiluba, C. Gortázar, Overcoming the limitations of wildlife disease monitoring, research directions, One Health 2 (2024), https://doi.org/10.1017/one.2023.16.
[45] D. Bass, K.W. Christison, G.D. Stentiford, L.S.J. Cook, H. Hartikainen, Environmental DNA/RNA for pathogen and parasite detection, surveillance, and ecology, Trends Parasitol. 39 (2023) 285–304, https://doi.org/10.1016/j.pt.2022.12.010.
[46] I.I. Zendoia, J.F. Barandika, A. Hurtado, C.M. López, E. Alonso, X. Beraza, B. Ocabo, A.L. García-Pérez, Analysis of environmental dust in goat and sheep farms to assess Coxiella burnetii infection in a Q fever endemic area: geographical distribution, relationship with human cases and genotypes, Zoonoses Public Health 68 (2021) 666–676, https://doi.org/10.1111/zph.12871.
[47] D. Jiménez-Martín, I. García-Bocanegra, M.A. Risalde, V. Fernández-Molera, S. Jiménez-Ruiz, J. Isla, D. Cano-Terriza, Epidemiology of paratuberculosis in sheep and goats in southern Spain, Prev. Vet. Med. 202 (2022), https://doi.org/10.1016/j.prevetmed.2022.105637.