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Mapping high-risk areas for *Mycobacterium tuberculosis* complex bacteria transmission: Linking host space use and environmental contamination

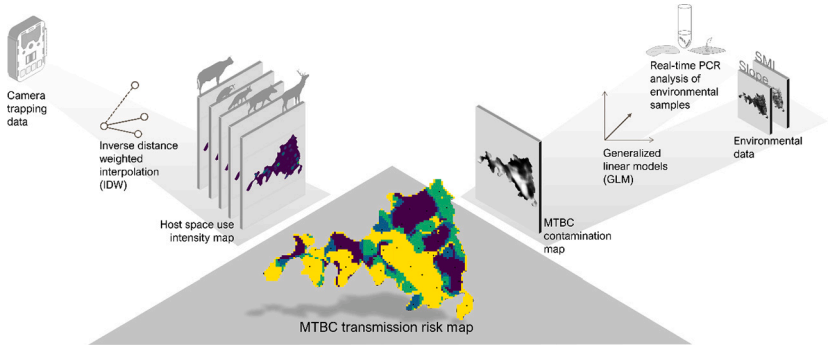
Eduardo M. Ferreira [a,b,c,*], Mónica V. Cunha [d,e], Elsa L. Duarte [a,f], António Mira [a,c], Daniela Pinto [d,e], Inês Mendes [d], André C. Pereira [d,e], Tiago Pinto [a,b,c], Pelayo Acevedo [g], Sara M. Santos [a,b,c]

[a] MED - Mediterranean Institute for Agriculture, Environment and Development & CHANGE – Global Change and Sustainability Institute, University of Évora, Mitra, 7006-554 Évora, Portugal
[b] IIFA - Institute for Advanced Studies and Research, University of Évora, Palácio do Vimioso, Largo Marquês de Marialva, 7002 - 554 Évora, Portugal
[c] Conservation Biology Lab, Department of Biology, University of Évora, Évora, Portugal
[d] Centre for Ecology, Evolution and Environmental Changes (cE3c) & CHANGE - Global Change and Sustainability Institute, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal
[e] Biosystems & Integrative Sciences Institute (BioISI), Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal
[f] Departamento de Medicina Veterinária, Pólo da Mitra, Apartado 94, 7002-554 Évora, Portugal
[g] Instituto de Investigación en Recursos Cinegéticos IREC (UCLM-CSIC-JCCM), Ronda de Toledo 12, 13071 Ciudad Real, Spain

HIGHLIGHTS

- The ecological background is seldomly incorporated in the investigation of non-vector-based disease systems.
- Host ecology and environmental contamination data were linked to quantify spatial transmission risk of MTBC in a multi-host system.
- Red deer and wild boar are associated to the highest-risk areas for MTBC transmission.
- One-quarter of the study area represents high transmission risk when considering a multi-host system.

GRAPHICAL ABSTRACT



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ABSTRACT

In many Mediterranean ecosystems, animal tuberculosis (TB), caused by *Mycobacterium bovis*, an ecovar of *Mycobacterium tuberculosis complex* (MTBC), is maintained by multi-host communities. It is hypothesised that interspecies transmission is mainly indirect via shared contaminated environments. Therefore, identifying spatial areas where MTBC bacteria occur and quantifying space use by susceptible hosts might help predict the spatial likelihood of transmission across the landscape. Here, we aimed to evaluate the transmission risk of MTBC in a multi-host system involving wildlife (ungulates and carnivores) and cattle (*Bos taurus*). We collected eighty-nine

* Corresponding author at: MED - Mediterranean Institute for Agriculture, Environment and Development & CHANGE – Global Change and Sustainability Institute, University of Évora, Mitra, 7006-554 Évora, Portugal.
E-mail addresses: ferreiraeduardo.mr@gmail.com (E.M. Ferreira), mscunha@ciencias.ulisboa.pt (M.V. Cunha), emld@uevora.pt (E.L. Duarte), amira@uevora.pt (A. Mira), dspinto@ciencias.ulisboa.pt (D. Pinto), tiagop@uevora.pt (T. Pinto), pelayo.acevedo@uclm.es (P. Acevedo), smsantos@uevora.pt (S.M. Santos).

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Space use

samples from natural substrates (water, soil, and mud) at 38 sampling sites in a TB endemic area within a Mediterranean agroforestry system in Portugal. These samples were analysed by real-time PCR to detect MTBC DNA. Additionally, host-specific space use intensity maps were obtained through camera-trapping covering the same sampling sites. Results evidenced that a significant proportion of samples were positive for MTBC DNA (49 %), suggesting that the contamination is widespread in the area. Moreover, they showed that the probability of MTBC occurrence in the environment was significantly influenced by topographic features (i.e., slope), although other non-significant predictor related with soil conditions (SMI: soil moisture index) incorporated the MTBC contamination model. The integration of host space use intensity maps with the spatial detection of MTBC showed that the red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*) exhibited the highest percentages of high-risk areas for MTBC transmission. Furthermore, when considering the co-occurrence of multiple hosts, transmission risk analyses revealed that 26.5 % of the study area represented high-risk conditions for MTBC transmission, mainly in forest areas.

1. Introduction

With the encroachment of human activities into wildlife habitats, an exponential growth of animal interactions across wildlife-livestock interfaces has been anticipated, with important implications in infectious disease emergence and transmission worldwide (Jones et al., 2013). Pathogens shared by wildlife and livestock have devastating consequences to livestock industry, biodiversity and public health (Webster et al., 2017; Hassell et al., 2021). Animal tuberculosis (TB), caused by *Mycobacterium bovis* or other ecovars of the *Mycobacterium tuberculosis complex* (MTBC), is one of the most prevalent and challenging health issues of cattle farming in many countries worldwide. In the European Union (EU), the eradication of TB in bovine has been a central priority. Despite all efforts made to date, some countries in the EU, including Portugal, have been unable to obtain the officially tuberculosis-free (OTF) status (Hardstaff et al., 2014; Pereira et al., 2020; Reis et al., 2020a; EFSA and ECDC, 2023). TB can persist in cattle farms due to interactions of cattle (*Bos taurus*) with several wildlife hosts that share the same areas and resources and are usually not under surveillance programmes (Varela-Castro et al., 2021; Herraiz et al., 2023). Specifically, some wildlife species that detain a significant role in TB epidemiology are considered reservoirs, maintaining the pathogen in ecosystems and transmitting infection to cattle, decreasing the success of eradication programmes (Duarte et al., 2008; Palmer, 2013; Canini et al., 2023; Gortázar et al., 2023).

Over the last years, numerous studies have been addressing TB dynamics and disease risk across different wildlife-cattle interfaces through varying ecological and epidemiological lenses (Acevedo et al., 2019; Pereira et al., 2023b). From an ecological perspective, different approaches (e.g., camera-trapping, proximity collars) have been used to characterise interaction networks within multi-host TB communities in farming systems (Kukielka et al., 2013; Drewe et al., 2013). The integration of ecological tools in TB epidemiology has improved our understanding of the likely transmission pathways of MTBC considering different eco-epidemiological scenarios (Wilber et al., 2019; Triguero-Ocaña et al., 2020a), with interactions between susceptible hosts being recognized as crucial determinants of transmission (Triguero-Ocaña et al., 2019; Ferreira et al., 2023).

Interaction patterns between susceptible hosts have been used as a reasonable proxy to discuss pathogen transmission risk, since it is very difficult to record real transmission events (e.g., Cowie et al., 2016; Campbell et al., 2019; Triguero-Ocaña et al., 2019). Nevertheless, pathogen transmission "*occurs at intersections of host and pathogen movement trajectories*" (Manlove et al., 2022). This means that pathogen transmission does not only occur directly when an uninfected susceptible host crosses paths with an infected host, but also indirectly when it comes into contact with the pathogen that has been spread in the environment by an infected host (Ferreira et al., 2023). In this sense, transmission risk depends not only on the frequency of interactions and host densities through space use, but also on the extent of environmental contamination (Dougherty et al., 2018). In fact, previous findings in different TB settings support the hypothesis that *M. bovis* transmission is mainly indirect through asynchronous use of contaminated shared environments by different host species (Allen et al., 2021; Triguero-Ocaña et al., 2021; Gortázar et al., 2023). An increased risk of transmission is expected during the dry season (the limiting season in Mediterranean environments) when natural food and water resources tend to become scarce, leading to the aggregation of multiple hosts at specific sites (e.g., supplementary feeding locations or at farm water sites) (Kukielka et al., 2013; Barasona et al., 2014). Despite the lower MTBC concentrations described in this season (Santos et al., 2015b), animals are more likely subjected to infection risks at spatially limited sites under harsh climate conditions (e.g., high temperature) where they might interact closely. A growing attention has been given to the potential role of the environment in animal TB epidemiology, particularly through attempts to evaluate the presence and distribution of MTBC in shared environments. Contamination of environmental substrates (e.g., water and soil) with MTBC DNA have been evidenced in various Mediterranean TB settings across the Iberian Peninsula (Santos et al., 2015b; Barasona et al., 2016; Pereira et al., 2023a; Herrero-García et al., 2024). Given the prolonged excretion of bacteria by infected hosts and viability of MTBC in the environment, animals might be exposed to increased risk for extended time periods (Vicente et al., 2013; Triguero-Ocaña et al., 2020a; Pereira et al., 2024). This is of particular concern in Mediterranean systems where MTBC is able to cause disease in multiple wildlife species (not only reservoir hosts) that occur in sympatry, along with cattle, originating complex multi-host communities (Santos et al., 2012; Matos et al., 2014; Gortázar et al., 2023).

Despite the evident need to close the gap between animal ecology and disease epidemiology fields, multi-disciplinary analyses are still uncommon, representing an under-explored avenue for investigation of disease systems at the wildlife-livestock interfaces (Dougherty et al., 2018; Manlove et al., 2022). To date, attempts to integrate ecological data on host space use with environmental exposure to TB remains scarce. Linking these components is crucial for accurately predicting the spatial likelihood of transmission risk in multi-hosts communities and identifying specific hotspot areas with high risk exposure (Barasona et al., 2016). Such knowledge can guide and refine disease control actions according to time-space-host axes in risk areas where disease persists. Hence, this work focuses on the transmission risk of MTBC during the dry season within a multi-host system involving wildlife (ungulates and carnivores) and cattle. We targeted a TB endemic area within a Mediterranean agroforestry system in Portugal with the following goals: 1) evaluate the extent of environmental contamination with MTBC; 2) identify environmental drivers influencing the occurrence of contamination and estimate the probability of MTBC occurrence across the landscape; and 3) predict potential high-risk areas for MTBC transmission, considering environmental contamination and host space use intensity.

2. Materials and methods

2.1. Study area

This study was carried out in Barrancos, located in southeast of Portugal (Alentejo region), near the Spanish border (38°08′ N; 6°59′ W) (Fig. 1). This area is recognized as a hotspot for TB in cattle and wildlife, and is included in the official epidemiological TB risk area where big game species (red deer [*Cervus elaphus*] and wild boar [*Sus scrofa*]) are the subject of a monitoring scheme that implies the initial examination in the field of hunted animals by a credentialed veterinarian to search for TB-compatible lesions (Cunha et al., 2011, DGAV, 2011, Santos et al., 2018). Ungulates are abundant in the region (wild boar density = 3–4 individuals/km^2; red deer density = 4–8 individuals/km2) (Santos et al., 2022). The dominant land use in Barrancos is the *Montado* (holm oak *Quercus rotundifolia* open woodland, with varying tree density) with extensive husbandry of cattle. Other land uses, albeit less abundant, include agricultural land, olive groves and scattered shrub areas. The topography is characterised by gentle to moderate undulating terrain, with altitude ranging between 160 and 350 m above sea level. The climate is Mediterranean, characterised by mild and wet winters and hot and dry summers. Mean winter temperatures (January) range from 5 °C to 14 °C, while mean summer temperatures (July) range from 15 °C to 34 °C (IPMA, 2023). The mean temperature during the research period was 25.5 °C in July.

Cattle herd TB prevalence was estimated at 1.83 % for the Alentejo region in 2022, higher than the national mean prevalence (Portugal mainland and Azores, 0.65 %) (DGAV, 2023). Also, during the field work (2021 to 2022), outbreaks were confirmed in Barrancos. Regarding wildlife, the few recent available studies point towards low TB rates (3.1 % and 1.8 % for red deer and wild boar, respectively; Costa, 2015) in Barrancos. However, at a national scale, a meta-analysis estimated the pooled TB prevalence as 27.5 % and 13.3 % for red deer and wild boar, respectively (Reis et al., 2020b).

2.2. Study design

We selected five free-ranging adjoining farms with similar management practices, comprising an area of ~3048 ha (farm size ranging from 148 ha to 980 ha), with an average of 136 adult cows per farm. A total of 38 sampling sites (Fig. 1) were defined: 16 water sites (natural water sources and water trough), three food sites (hay feeders), and 19 control sites (without any water sources or supplementary food, e.g., forest animal path). Minimum distance between sampling sites averaged 686 m (range: 350 m to 1300 m). These sites were defined and sampled with camera traps for monitoring animal visitation and interaction rates within a previous work (see below the description on the inference of transmission risk maps; more details in Supplementary material: Camera-trap design).

A total of 89 environmental samples were collected in September 2022 (dry season) (two samples were collected at each control site; and two to three samples were collected at each water/food sites). Samples included water (n = 10), mud (n = 17) and soil (n = 62). They were collected into sterile propylene flasks (1000 mL) and kept at 4 °C during transportation. Samples were then frozen until laboratory analysis were performed.

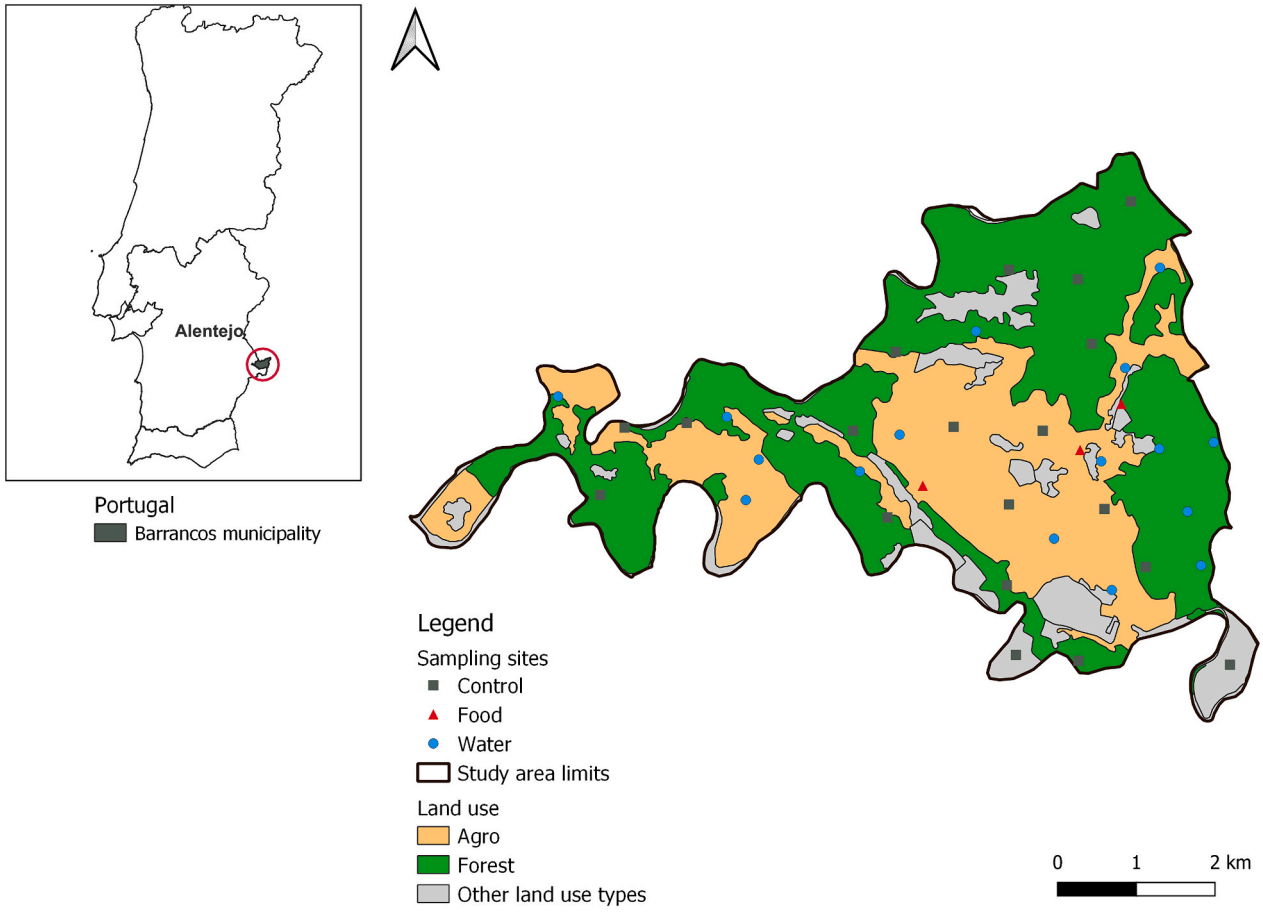

Fig. 1. Study area location in Barrancos region, Portugal, showing sampled sites and main land uses. Agro: holm oak stands with low or absent shrub cover due to grazing and other pastoral activities; Forest: holm oak stands or mixed woodland patches with high shrub cover.

2.3. Sample processing, DNA extraction and MTBC detection by qPCR

Samples were processed and analysed as described in Pereira et al. (2023a). Briefly, collected mud and soil samples were subjected to homogenization by stirring. Subsequently, 250 g of each sample were resuspended in 50 mL of cell recovery solution, comprising 1× PBS, 0.05 % Tween®80, and 0.01 % sodium pyrophosphate, and incubated at 28 °C for 30 min with continuous shaking. Following incubation, the sample suspensions underwent centrifugation at 150 ×*g* for 5 min and the supernatant was collected. For collected water samples, a 10 μm pore size filter was employed, and the resulting filtrate was centrifuged at 3220 ×*g* for 30 min. The cell pellet obtained was then resuspended in 10 mL of 1× PBS. Processed, resuspended soil and water were centrifuged at 3220 ×*g* for 30 min. The supernatants were discarded.

DNA extraction was conducted using 250 mg of sediments from the previous step and the DNeasy PowerSoil Pro kit (Qiagen, USA), adhering to the manufacturer's instructions. Subsequently, DNA quantification was performed using Qubit™ dsDNA Quantification Assay Kits (ThermoFisher Scientific), following the manufacturer's guidelines. The presence of MTBC was assessed through molecular methods, namely using real-time PCR with IS*6110*-specific primers and probe, as previously described (Costa et al., 2014). IS*6110* is the most common PCR target for MTBC detection, being a widely used marker in epidemiological studies (Costa et al., 2014, Pereira et al., 2023a). In brief, NZY-Supreme qPCR Probe Master Mix (NZYtech, Portugal) was utilized along with 0.4 μM of each primer and 0.2 μM of the probe. Five microliters of 10-fold diluted total DNA were added to the reaction mix. Amplification consisted of an initial denaturation step of 3 min at 95 °C, followed by 45 cycles of 5 s of denaturation at 95 °C and 30 s of extension at 60 °C. Thermal cycling and fluorescent signal acquisition occurred in a Bio-Rad CFX96 thermocycler (Bio-Rad, USA), with reactions performed in triplicate.

Negative results were confirmed by testing 5 μL of undiluted samples to detect low MTBC burden. Reactions were initially performed in duplicate for all samples, except in cases of disagreement between duplicates, where a triplicate was performed. It is noteworthy that *M. bovis* BCG Pasteur has a single copy of IS*6110*; however, other members of the MTBC may possess up to 16 copies (Comín et al., 2022). Positive, negative, and blank controls were included in each PCR batch.

2.4. MTBC occurrence: predictor selection

We considered a total of 16 environmental predictors that might explain the occurrence of MTBC in environmental matrices from the study area (Table 1) (Walter et al., 2014; Martínez-Guijosa et al., 2020; Allen et al., 2021; Pereira et al., 2023a).

Regarding topographic predictors, we estimated elevation from a 30-m Digital elevation Model (DEM), and derived slope and hillshade metrics from the DEM using Quantum GIS v. 3.0.3 (QGIS, 2022). For landscape composition-related predictors, we computed the percentage of land cover, considering the main land uses (Agro and Forest) occurring in the study area. The Shannon landscape diversity index and the Euclidean distance of sampling sites to forest edges were also computed. Those metrics were obtained from the Corine Land Cover (2018) dataset (European Union, Copernicus Land Monitoring Service, European Environment Agency) and were retrieved from the 'landscapemetrics' R package (Hesselbarth et al., 2019). In addition, tree cover density was derived from the Tree Cover Density (2018) dataset (Copernicus Land Monitoring Service, European Environment Agency). Remote-sensing data were derived from the LANDSAT 8 image collection (level 2, Tier 1) to the period of sample collection (September 2022), with a 30 m spatial resolution, and processed in Google Earth Engine (Gorelick et al., 2017). Only high-quality images (with ≤5 % of cloud cover) were considered (Pinto et al., 2023). Soil texture predictor variables were extracted from the European Soil Data Centre (ESDAC, http://esdac.jrc.ec.europa.eu/; Panagos et al., 2012). The Shannon wildlife diversity index was calculated using the visitation rates (the number of detections of each species at each sampling site in a month/(number of active camera days/number of days of a given month)) of the target species derived from camera trap monitoring, considering averaged values for the dry season.

A multi-scale modelling approach was carried out to maximise accuracy of predictors. Continuous predictors not based on distances (Altitude, Slope, Hillshade, Agro, Forest, TreeD, LST, SMI, EVI, NDWI) were stacked in a 30 m spatial resolution multi-raster layer. We then applied the following spatial scales of analysis: 90, 240 and 510 m focal-radius moving window as a proxy for 100, 250 and 500 m scales of analysis (Ferreira et al., 2024). Mean was used to summarize the raster values within each spatial scale.

Table 1
Description of the environmental predictors used for modelling the occurrence of MTBC.

Predictor group	Predictor code	Predictor type	Description	Original spatial resolution
Topography	Altitude	Numeric	Terrain altitude within 100, 250 and 500 m spatial scales around sampling sites (m).	30 m
	Slope	Numeric	Topographic slope within 100, 250 and 500 m spatial scales around sampling sites (degrees).	30 m
	Hillshade	Numeric	Hillshade – light reflectance from a terrain surface – expressed within 100, 250 and 500 m spatial scales around sampling sites (degrees).	30 m
Landscape composition	Agro	Numeric	Percentage of agroforest land (holm oak stands with low or absent shrub cover due to grazing and other pastoral activities) within 100, 250 and 500 m spatial scales around sampling sites (%).	
	Forest	Numeric	Percentage of forest (holm oak stands or mixed woodland patches with high shrub cover) within 100, 250 and 500 m spatial scales around sampling sites (%).	
	Dist_ForestEdge	Numeric	Distance of sampling sites to the nearest edge of forest patches (m).	30 m
	TreeD	Numeric	Percentage of tree cover density within 100, 250 and 500 m spatial scales around sampling sites (%).	30 m
	Dist_waterlines	Numeric	Distance of camera sites to the nearest water line (m).	30 m
Abiotic components (remote sensing)	LST	Numeric	Radiative temperature of land surface (°C) within 100, 250 and 500 m spatial scales around sampling sites.	30 m
	SMI	Numeric	Soil moisture index within 100, 250 and 500 m spatial scales around sampling sites.	30 m
	EVI	Numeric	Enhanced Vegetation Index within 100, 250 and 500 m spatial scales around sampling sites.	30 m
	NDWI	Numeric	Normalised Difference Water Index within 100, 250 and 500 m spatial scales around sampling sites.	30 m
Soil texture	Clay	Numeric	Percentage of soil clay expressed in a 500 m spatial scale around sampling sites (%).	500 m
	Silt	Numeric	Percentage of soil silt expressed in a 500 m spatial scale around sampling sites (%).	500 m
	Sand	Numeric	Percentage of soil sand expressed in a 500 m spatial scale around sampling sites (%).	500 m
Ecological factors	Wild_div	Numeric	Wildlife diversity at sampling sites.	

2.5. Ecological modelling of MTBC

We modelled MTBC DNA presence in environmental samples to identify drivers of environmental contamination and predict patterns of transmission risk. We calculated the proportion of positive samples per sampling site (*prop*) by dividing the number of positive samples by the total number of samples analysed for each site. Afterwards, we defined a binomial response variable (MTBC occurrence; *occurrence_bin*) based on *prop* to be used in modelling. When prop was ≥0.5, we considered a sampling site as potentially contaminated (coded as 1; $n = 24$); otherwise, the site was considered non-contaminated (coded as 0; $n = 14$). Generalized linear models (GLMz) were applied to test the effects of predictors on MTBC occurrence. These models were chosen for their suitability for binary prediction and frequently used for disease mapping (de Oliveira et al., 2022; Li et al., 2022; Ndolo et al., 2022).

We first ran univariate models to identify likely relevant predictors. As such, fitted univariate models testing one predictor at a time were compared with the null model using AICc, Akaike's Information Criterion adjusted for small sample sizes (Burnham and Anderson, 2002). A predictor variable was considered informative when: 1) the 95 % confidence intervals (CI 95 %) of the predictor coefficient being tested did not include zero; and 2) a delta AICc >2 was obtained when comparing the univariate model with the null model (Burnham and Anderson, 2002; Stephens et al., 2005). If highly correlated informative predictors ($r > |0.7|$) were identified, we only retained the one producing a lower AICc to be included in the multivariate model. Multivariate models were built testing all possible combinations of the informative predictors using dredge in the 'MuMIn' package (Bartoń, 2022). When several models had ΔAICc <2, all associated predictors were included in a single best multi model (e.g. Humphrey et al., 2023). Prediction performance of the best model was assessed using the area under the curve (AUC) of the receiver operating characteristic [ROC], combined with model accuracy and Cohen's kappa coefficient through Leave One Out Cross Validation (LOOCV) procedure (Morris et al., 2016; Xia et al., 2019; Deka, 2022). We obtained the potential occurrence of MTBC in the study area by projecting the best model to the entire study area.

2.6. Development of transmission risk maps

To evaluate the transmission risk for each animal host, two components were combined: (1) the potential occurrence of MTBC in the study area and (2) host-specific space use intensity maps. Space use intensity maps for each host (cattle, wild boar, red deer, red fox [*Vulpes vulpes*], and badger [*Meles meles*]) were obtained on the same sampling sites (Ferreira et al., 2024). For each of the sampling sites, a camera-trap was installed during the dry season 2021 (June to September; Kukielka et al., 2013, Cowie et al., 2016) to measure visitation rates by domestic cattle and wildlife species. Busnhell Trophy Cam HD Aggressor or Reconyx Hyperfire cameras were used and placed 30–50 cm above the ground. No bait of any kind was used. We programmed cameras to operate 24 h a day, taking three pictures per trigger with a 30-second delay between consecutive triggers (Kukielka et al., 2013; Triguero-Ocaña et al., 2020b). Visitation rates were calculated for each sampling site and each host species, considering 15 min as the time to independent observations (Kukielka et al., 2013; Carrasco-Garcia et al., 2016; Martínez-Guijosa et al., 2021). Visitation rates were first calculated as the number of detections of each species at each sampling site in a month/(number of active camera days/number of days of a given month). We then calculated the mean visitation rate (VR), discriminated by species, for the dry season at each sampling site by averaging visitation estimates across all sampled months (see Supplementary material: Host space use intensity maps and Table S1).

Species-specific space use intensity maps were generated based on averaged visitation rates by inverse distance weighted interpolation (IDW) (e.g., Sarmento et al., 2011; Curveira-Santos et al., 2019), thus producing spatial interpolation surfaces for the entire study area. We tested different combinations of IDP (inverse distance power) and nmax (the number of nearest observations for prediction) values. The chosen values were based on a balance between statistical accuracy (lower RMSE [Root Mean Square Error]) and spatial coherence (considering land-uses and species ecological traits [e.g., minimal vital areas]). Predicted space use intensity maps for each species are available in the Supplementary material, Figs. S1–S5.

We reclassified the host space use intensity maps using quartile intervals as follows: low (VR < 2Q, i.e., second quartile), medium (2Q ≤ VR < 3Q) and high (VR ≥ 3Q). Similar reclassification was applied to MTBC contamination map based on the predicted probability, grouping it into three categories: low (MTBC occurrence probability <0.5), medium (MTBC occurrence probability ≥0.5 & <0.75) and high (MTBC occurrence probability ≥0.75). After, transmission risk maps were built for each target host species based on reclassified MTBC contamination and host space use intensity maps. A high-risk transmission level was assigned to a given area when both maps indicated high conditions, or when high and medium conditions were combined (Supplementary material: Table S2). Areas classified as medium-risk resulted from either the convergence of two medium conditions or the combination of high and low conditions. The remaining areas were designated as low-risk transmission using similar criteria, based on the intersection of low with medium conditions, and low with low conditions. A final multi-host transmission risk map was also generated by overlaying high-risk transmission areas shared between wildlife and cattle hosts using R packages 'raster' and 'geoR' (R Core Team, 2022). Specifically, a combined map was built based on high-risk areas considering a gradient of hosts: areas associated with just one host, two hosts, and with three or more hosts, serving as a proxy for multi-host TB scenarios.

3. Results

3.1. Environmental contamination with MTBC

From a total of 89 samples collected across 38 sites, 49 % were positive for the presence of MTBC DNA. Similar percentage of positive samples were registered for mud (53 %) and soil (56 %) matrices. No positive samples (0 %) were recorded for water matrices. Hence, considering MTBC occurrence, 63 % of sampling sites were considered contaminated by MTBC, with 29 % having all samples testing positive for the presence of MTBC.

3.2. Environmental drivers influencing MTBC contamination

The MTBC contamination GLM model had good fit, with an estimated accuracy of 0.79 and a Kappa value of 0.55. Additionally, it had good discriminating ability (AUC = 0.82). The best model included Slope (scale 250 m) and SMI (scale 500 m) as predictor variables. Slope had a positive and significant effect on the probability of MTBC occurrence (coef = 1.409, CI 95 % [0.156; 2.662]). A positive relation was also detected between SMI and MTBC occurrence, although not statistically significant (coef = 0.478, CI 95 % [−0.561, 1.518]). According to the predicted map, 26.9 % of the study area is categorized as low risk for MTBC occurrence (occurrence probability <0.5) while 73.1 % is considered as medium to high risk (occurrence probability ≥ 0.5) (Fig. 2). The high-risk areas for MTBC occurrence (49 % of the study area) occur across all the SA but are predominantly concentrated in the Northeast section.

3.3. Prediction of potential high-risk areas for MTBC transmission

Overall, transmission risk analysis revealed that the SA is dominated by medium-risk areas for MTBC transmission (43 % of the study area extension; SD = 8). Low and high-risk areas came in second, equally represented, each comprising 29 % of the study area (SD = 4 and 5, respectively) (Table 2).

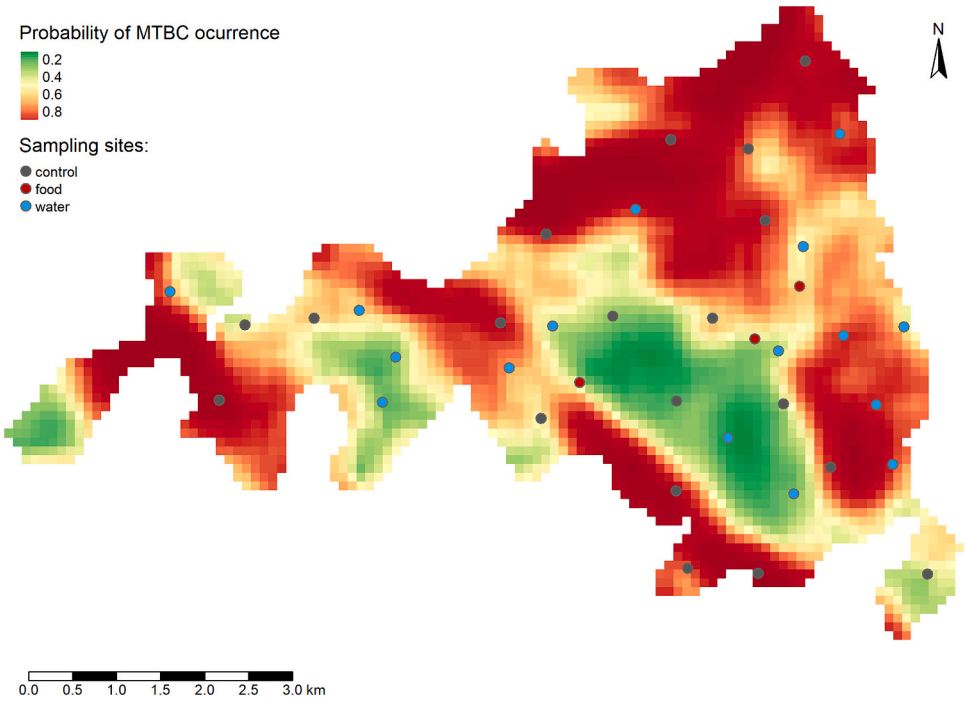

Fig. 2. Risk map of MTBC occurrence in the study area, Southeast of Portugal, layered with sampling sites.

Table 2
Percentage of area occupied by low, medium, and high-risk areas for MTBC transmission in the study area according to the target hosts (cattle, wild boar, red deer, red fox and cattle).

Host species	% low risk areas	% medium risk areas	% high-risk areas
Cattle	25.2	50.7	24.1
Wild boar	29.3	39.4	31.3
Red deer	34.4	30.2	35.4
Red fox	27.3	46.6	26.1
Badger	26.6	47.6	25.8
mean	28.5	42.9	28.5
sd	3.6	8.2	4.7

The red deer and the wild boar were associated with the highest percentage of high-risk areas (Table 2, Figs. 3 and 4, respectively). Transmission risk maps for these species exhibited similar spatial trends, with the main high-risk areas concentrated in the southeast, north, as well as in the west-central sections of the SA. Although less represented (see Table 2), high risk areas for cattle shows a substantial degree of overlap with wild ungulates high-risk transmission areas, particularly in the east and west-central sections, albeit with slightly different spatial configurations (Fig. 5). High-risk areas for MTBC transmission associated with red fox and badger are concentrated in the northern section of the SA (Figs. 6 and 7, respectively). Smaller and more fragmented high-risk areas are present in the western section of the SA as well. Much of these areas are concentrated in forest areas, but covering small portions of agro land use, and including water, control, and food sites.

When examining the overlap of high-risk areas across various host settings (involving one, two or more), results indicated that 26.5 % of the SA is designated as high-risk when considering multi-host conditions (Fig. 8). There are three main core areas of high-risk distributed along the southeast to northeast axis, with two additional areas located in the western section of the SA. High-risk areas involving multi-host conditions included five water sites (5/16; 31 %), one food site (1/3; 33 %), and two control sites (2/19; 11 %). Furthermore, the transmission risk map indicated that 18.9 % of the SA poses a high-risk for MTBC transmission for a single TB host, whereas only 11 % are deemed high-risk when considering two hosts combined.

4. Discussion

Incorporating data on host space use into disease models can improve predictions of transmission dynamics, thus aiding in the definition of priority areas for effective disease control (Morris et al., 2016; Dougherty et al., 2018). While the complex interplay between host ecology and transmission pathways (direct and indirect) for animal TB has been studied in some depth (Payne et al., 2016; Varela-Castro et al., 2021), significantly less is known on how host ecology and spatial gradients of MTBC occurrence influence transmission risk across the landscape.

In this study, we demonstrated that: 1) environmental contamination with MTBC is widespread in different types of environmental matrices in the study area; 2) the probability of MTBC occurrence significantly increased in areas with higher slope values; 3) transmission risk analyses provided valuable insights into the spatial distribution of high-risk areas associated with different MTBC hosts. Red deer and wild boar presented the highest percentages of high-risk transmission areas, with a significant overlap with cattle-related areas. Furthermore, results suggested that a substantial proportion of the study area (26.5 %) could be at high-risk when considering the co-occurrence of multiple hosts. Regardless of the host considered, high-risk areas are primarily concentrated in forest areas (dense shrub cover) but also encompass small portions of agro land use (reduced or absence shrub cover). They not only include recognized aggregation points, such as water and artificial food sites, but also encompass control sites (e.g., random sites such as animal trails, pastures) where animal encounters are less likely to occur.

4.1. Environmental contamination with MTBC

In our study area, a total of 49 % of tested samples were positive for the presence of MTBC DNA and 63 % of sampling sites were deemed contaminated. This pattern is in agreement with other recent studies conducted in epidemiological risk areas across the Iberian region, also characterised by multi-host communities. In Idanha-a-Nova, nearby the International Tagus Natural Park region (Portugal), Pereira and

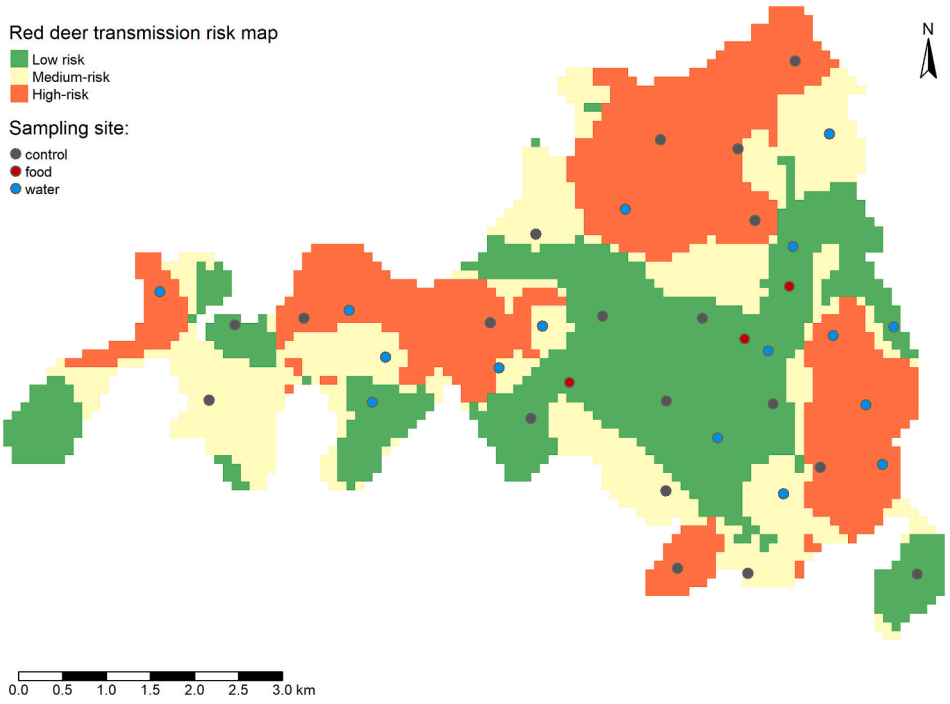

Fig. 3. MTBC transmission risk map for red deer in the study area, Southeast of Portugal, layered with sampling sites.

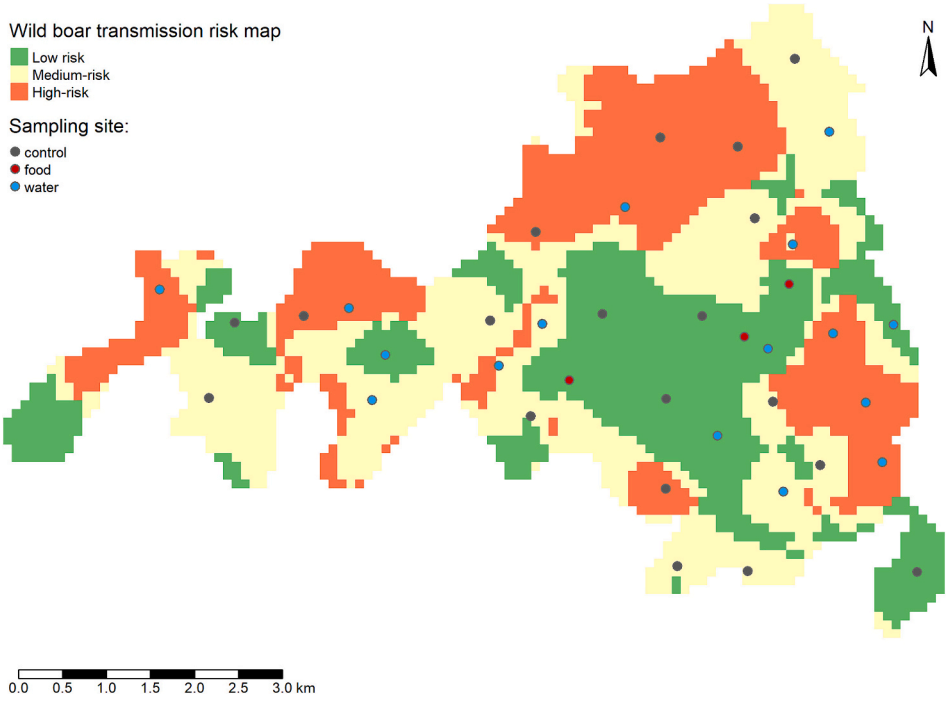

Fig. 4. MTBC transmission risk map for the wild boar in the study area, Southeast of Portugal, layered with sampling sites.

colleagues found that the majority of samples (54 %) contained metabolically active or dormant MTBC cells (Pereira et al., 2023a). Similarly, in the Alentejo region, Santos et al. (2015b) confirmed the widespread environmental contamination with MTBC in a TB infected area, with 32 % of samples testing positive for MTBC DNA. In Spain, where wildlife-cattle interfaces share many ecological and environmental characteristics with Portugal ecosystems, up to 55.8 % of sampling sites – mud samples collected at water sites – tested positive for MTBC DNA (Barasona et al., 2016). Additionally, studies conducted elsewhere also demonstrated the occurrence of MTBC in the environment (i.e., badger setts and latrines in cattle farms), such as in the UK, a non-officially free country where badgers are considered a reservoir host (Courtenay et al., 2006).

In our study, three types of environmental matrices were examined, with higher rates of positivity recorded in mud and soil matrices (56 % and 53 %, respectively), whereas MTBC DNA was absent from water samples. Barasona et al. (2016) and Pereira et al. (2023a) also recorded higher rates of positivity in mud samples collected from water sites (48

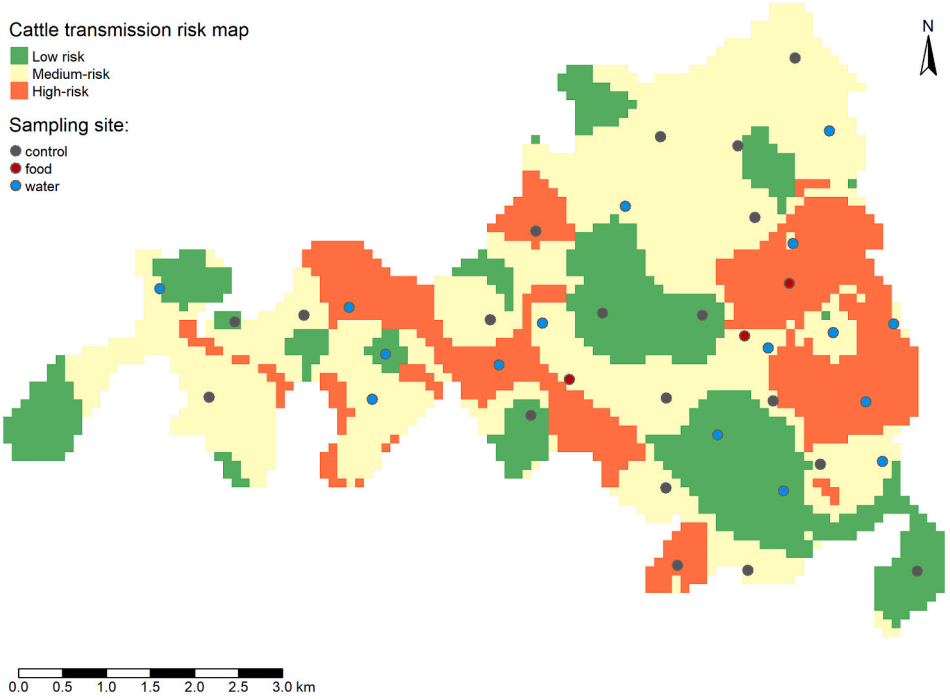

Fig. 5. MTBC transmission risk map for cattle in the study area, Southeast of Portugal, layered with sampling sites.

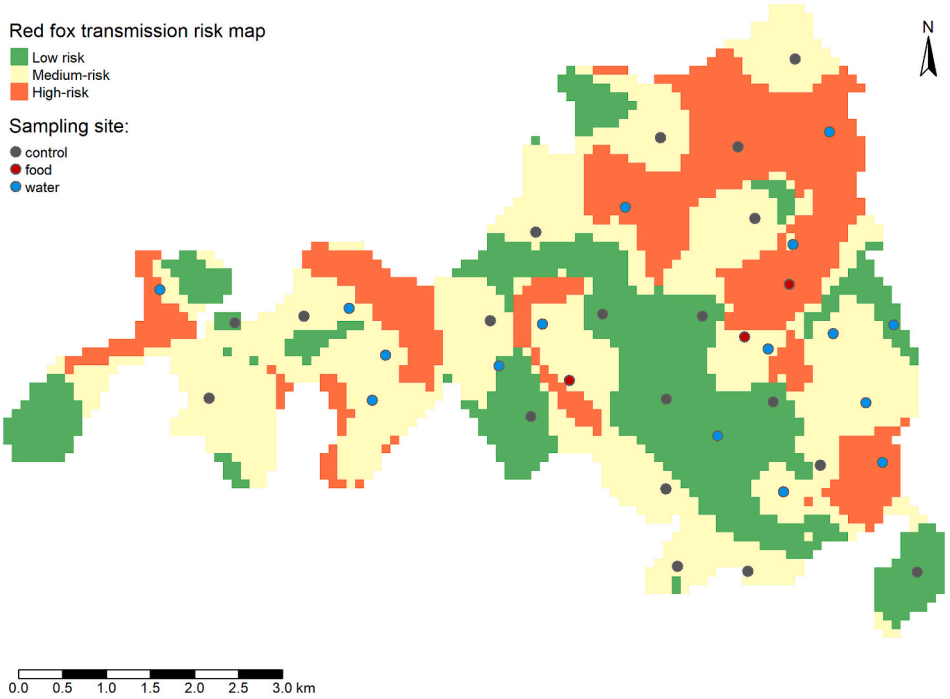

Fig. 6. MTBC transmission risk map for red fox in the study area, Southeast of Portugal, layered with sampling sites.

% and 53 %, respectively). Contrary to our findings, they detected MTBC DNA in water samples, albeit lower proportions of positive samples were recorded (ranging between 8.9 % and 19 %). Similarly, Santos et al. (2015b) documented significantly lower positivity rates in water samples from dams when compared to other sample types, regardless of the season. Nevertheless, water sites, and even running water, could become contaminated with MTBC from cattle or from wildlife excretions, and thus also constitute an infection source (Allen et al., 2021). In addition, this is likely to be a relevant issue during the dry season, when various species aggregate around limited water sites as described in shared interfaces across Mediterranean environments (Kukielka et al., 2013; Triguero-Ocaña et al., 2019). On the other hand, given the lower rates of positivity in water samples, we hypothesised that surface water is unlikely to be significant in the transmission of MTBC in this ecosystem, in opposition to mud samples. Overall, reported MTBC prevalence rates in mud samples tend to be high (around 50 %) in TB multi-host systems (Barasona et al., 2016; Pereira et al., 2023a). Additionally, high prevalence rates of MTBC/*M. bovis* have been detected in sediment samples,

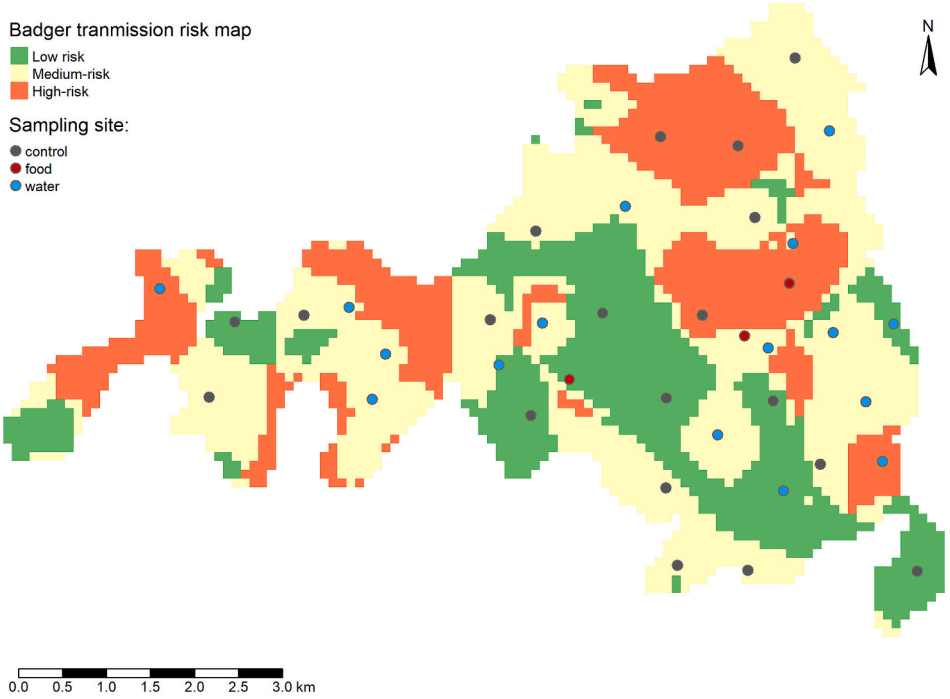

Fig. 7. MTBC transmission risk map for badger in the study area, Southeast of Portugal, layered with sampling sites.

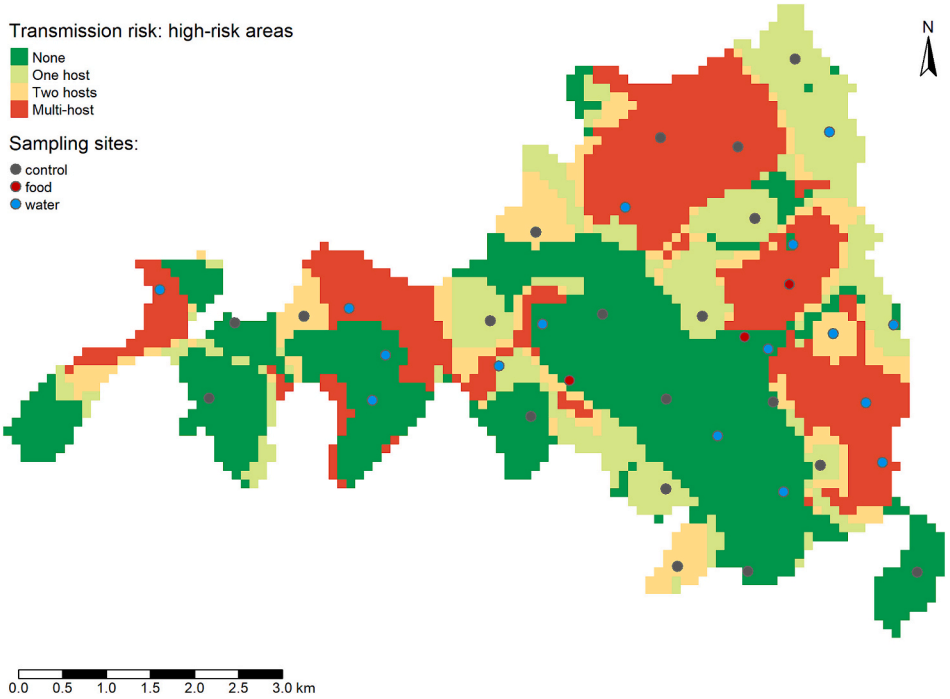

Fig. 8. MTBC multi-host transmission risk map covering high risk-areas according to different host species compositions, including a multi-host scenario, in Southeast of Portugal, layered with sampling sites.

although marked heterogeneity was observed, depending on the sediment type, study system and season (Santos et al., 2015b; Martínez-Guijosa et al., 2021).

Regardless of the sample type considered, host space use intensity and host behaviour are likely key factors that render a given site more prone to MTBC contamination and persistence. Sites that are more attractive to numerous wild species are expected to be at a higher risk of contamination with MTBC because more animals may shed MTBC into the environment. Furthermore, host behaviour might influence the length of contact time with the environment and the number of pathogens shed. For example, wild boar and even red deer tend to wallow in water sites (Carrasco-Garcia et al., 2016), leading to prolonged and significant physical contact with the environment. This may increase the likelihood of environmental contamination, particularly in sediments-like mud, due to the excretion from infected animals that tends to occur through various routes (e.g., oronasal, urinary) (Santos et al.,

2015a; Barasona et al., 2017).

4.2. Slope and soil moisture index are predictors of MTBC environmental contamination in the study area

When analysing environmental drivers of MTBC occurrence, the GLMz model indicated that the probability of occurrence in the SA was positively related with slope and soil moisture index, yet only slope demonstrated a significant effect. Although the drivers of MTBC occurrence across spatial scales is still a relatively poorly studied topic, other authors have also shown connections between environmental features and the presence of MTBC in Iberian contexts. For instance, Martínez-Guijosa et al. (2020) demonstrated a greater risk of detecting MTBC DNA on farms at higher altitudes. We hypothesised that areas with pronounced slopes – mainly associated with forests in the study area – may feature specific conditions (e.g., greater heterogeneity of shadows, moist conditions, and humidity) that could reduce the effects of extreme temperatures and direct sunlight. These factors are known to be critical for MTBC survival (Rodríguez-Hernández et al., 2016; Barbier et al., 2017; Allen et al., 2021). Previous studies have also demonstrated that topography-related factors were important in predicting the abundance distribution of soil bacteria and even bacterial community composition (Liu et al., 2020; Mod et al., 2021). Our findings highlight the need to account for topographic and also edaphic factors in future forecasts of MTBC occurrence, as specific environmental requirements (e.g., niches) are still being uncovered.

We found no support for the effect of other tested predictors on MTBC occurrence. However, recent studies have demonstrated that, depending on the disease-ecological system, MTBC occurrence can be affected by land use factors (Pereira et al., 2023a), configuration of water sites, soil-related factors (e.g., soil temperature) (Santos et al., 2015b) and the presence of wildlife cachectic animals (Barasona et al., 2016). Future studies should aim to encompass larger sample sizes across diverse geographical areas, explore different sets of potential drivers, and assess the metabolic state of MTBC. This can offer new opportunities to identify specific environmental signatures related with MTBC, thereby improving predictive accuracy of modelling approaches and refining infection risk assessments.

4.3. Prediction of potential high-risk areas for MTBC transmission confirms the central role of red deer and wild boar on TB epidemiology

Wild boar and red deer are considered the most important animal TB reservoir hosts in the Iberian Peninsula: infection is maintained in eco-epidemiological scenarios where any of these species acts as a single reservoir (most often the wild boar) to a facultative multi-host situation (Gortázar et al., 2012; Santos et al., 2022). Our transmission risk analyses demonstrated that red deer and wild boar presented the highest percentages of high-risk transmission areas, thereby supporting their key role on TB epidemiology in the study area. This hypothesis can be supported by three main premises: first, high abundance of wild ungulates that coexist in the same space. Animal density is recognized as a key element in pathogen transmission (Manlove et al., 2022). In our case, both species are highly abundant in the study area – as reported in other regions across the central-southwestern section of the Iberian Peninsula – and thus likely enhance pathogen transmission and maintenance (Vicente et al., 2013; Santos et al., 2022); second, specific ecological traits in terms of space use can favour transmission in shared environments. Results of camera-trapping surveys support previous findings that both species extensively explore a variety of spatial sites (Laguna et al., 2021b, 2021a). However, a significant proportion of high-risk areas (for both species) is associated with forest areas (indicated by higher slope values), being predominantly concentrated in the Northeast section of the study area. This crucial aspect proves that two main conditions are linked: areas that may provide favourable conditions for pathogen survival (e.g., higher shade in stepper areas) and spatial sites frequently used by ungulates. Therefore, hosts with territories encompassing these characteristics, are expected to foment environmental contamination and have a higher transmission risk; third, wild boar and red deer populations include "super-shedders" individuals. These can develop extensive lesions and excrete considerable amounts of mycobacteria through several routes, occurring intermittently from early stages of the disease (Santos et al., 2015a). In this regard, ungulates are central hosts that could influence environmental contamination and within-host persistence in Mediterranean multi-host systems, as we hypothesised in our study. Consequently, cattle, by sharing areas with ungulates (e.g. ecotone zones between forest and agro land uses), can be exposed to an increased infection risk.

High-risk areas for MTBC transmission associated with red fox and badger, overall, exhibit similar patterns to those of ungulates when considering their spatial distribution. However, high-risk areas are smaller and more fragmented, and particularly less prevalent in the eastern section of the study area. In our study, carnivores displayed a higher intensity of space use more frequently in spatial sites located in agro land use, rather than deep within forest areas. A similar and more pronounced pattern can be observed with cattle, which tend to avoid large, forest patch areas. As a result, transmission risk maps do not designate the northern area of the study area (the largest contiguous forest area) as high-risk, in opposition to wild hosts. Regardless of the host considered, it should be noticed that medium-risk areas for MTBC transmission were the most dominant in the study area. Nevertheless, when considering potential control measures *in situ* to target multiple hosts in complex communities, the identification of critical areas (high-risk areas) should be a priority (Barasona et al., 2013; Triguero-Ocaña et al., 2019; Gortázar et al., 2023). Decisions about where to act (e.g., site selection) are challenging when considering varying transmission risk gradients that arise from distinct ecological backgrounds of hosts (De Garine-Wichatitsky et al., 2021). We took a further step in this direction by identifying transmission risk areas across multiple hosts. Twenty-six-point 5 % of the SA is considered high-risk for MTBC transmission when considering multi-host conditions. Accordingly, there are five main core areas primarily associated with forest areas but also encompassing marginal portions of open areas (e.g. agro land use). Disease control measures should focus on these areas, encompassing specific spatial sites (e.g., artificial food sites and water sites) that tend to promote host aggregation, but also natural areas (e.g., pastures) widely distributed across the landscape.

5. Conclusions

Our findings quantified transmission risk gradients in a TB multi host system involving ungulates, carnivores, and cattle. Our predictions, by combining host space use maps with the spatial occurrence of MTBC, provide, for the first-time, risk maps useful for targeting priority areas for MTBC surveillance and control. We demonstrated the presence of MTBC in the environment, specifically in soil and mud matrices, wherein topographic features (i.e., slope) may play a key role. Although wild boar and red deer presented the highest percentages of high-risk areas regarding MTBC transmission risk, our results indicated a potential for high-risk areas when considering the co-occurrence of multiple hosts. Thus, management of disease within multi-host systems may require focusing on such areas, as pathogen abundance depends on the cumulative presence of all relevant hosts involved. Our approach can be applicable to other disease systems that are likely mediated through shared environments, informing and guiding risk assessment plans for control and management actions.

CRediT authorship contribution statement

Eduardo M. Ferreira: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mónica V. Cunha:** Writing – review &

editing, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Elsa L. Duarte:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **António Mira:** Writing – review & editing, Validation, Resources, Funding acquisition. **Daniela Pinto:** Writing – review & editing, Investigation. **Inês Mendes:** Writing – review & editing, Investigation. **André C. Pereira:** Writing – review & editing, Investigation. **Tiago Pinto:** Writing – review & editing, Data curation. **Pelayo Acevedo:** Writing – review & editing. **Sara M. Santos:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

The data associated with this research are available from the corresponding author upon reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.176053.

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