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Modeling the transmission risk of *Mycobacterium avium* subsp. *paratuberculosis* across the mammal community of a Mediterranean agro-forestry farmstead

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ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiological agent of paratuberculosis, a chronic infection affecting ruminants worldwide. In wildlife, MAP was first detected in the European rabbit and has since then been reported in a very broad host range. Information on the ecological factors that increase infection risk, as well as evidence for the transmission paths linking livestock, wildlife, and the environment, remain scarce. Thus, the objectives of this thesis included estimating MAP prevalence in the mammal community of a Mediterranean agro-forestry farmstead, the Companhia das Lezírias, through field and wet lab approaches, assessing MAP's spatial distribution, determining which factors modulate exposure of wildlife to MAP, and predicting MAP risk within the study area. Using molecular detection of IS*900* as a proxy, MAP was detected in ten wild mammal species, with emphasis on wild rabbit (19% overall prevalence), in cattle (54% individual prevalence and 100% herd prevalence) and in soil (44% prevalence). Wildlife diversity showed a positive influence on MAP presence in mammal feces, while wildlife abundance showed a negative influence. Land use variables showed distinct degrees of influence on MAP presence in feces of specific groups of mammals: mixed forest showed positive influence in carnivores, and shrubland showed positive influence in wild rabbit. The spatial prediction of MAP occurrence risk in wildlife generated two hotspots; however, model accuracy was low. In conclusion, the variables considered were insufficient to accurately predict MAP occurrence risk in mammals in our study area, showing the need for further studies. Increasing the number of samples and sampled species, as well as considering new variables, could improve prediction accuracy. Despite these limitations, this study represents a significant step forward in the knowledge of MAP occurrence at the livestock-wildlife interface in a Mediterranean agroecosystem.

Keywords
Livestock-wildlife-environment interfaces; mammals; *Mycobacterium avium* subsp. paratuberculosis; transmission

RESUMO

Mycobacterium avium subsp. *paratuberculosis* (MAP) é o agente etiológico da paratuberculose ou doença de Johne, uma infeção crónica que afeta principalmente o sistema gastrointestinal. Esta doença pode causar diarreia, edema submandibular, perda de peso, desnutrição, anemia, letargia e, em casos mais graves, morte. No entanto, os animais podem permanecer assintomáticos durante dois a cinco anos. Os portadores assintomáticos não detetados podem contribuir significativamente para a transmissão de MAP, visto que excretam bactérias através do leite e dos dejetos, promovendo a infeção de outros animais e a contaminação do ambiente circundante.

Os ruminantes domésticos parecem ser os hospedeiros preferenciais de MAP; no entanto, a infeção em ruminantes selvagens está também bem documentada. Para além de ruminantes, a presença de MAP foi detetada em coelho-bravo (*Oryctolagus cuniculus*) pela primeira vez na Escócia. Desde aí, vários estudos reportaram infeção num grande número de hospedeiros, incluindo aves e diversos *taxa* de mamíferos, como lagomorfos, carnívoros, roedores, ungulados e primatas. Também já se isolou MAP em invertebrados, como insetos e minhocas.

Continua a haver pouca informação quanto aos fatores que aumentam o risco de infeção, assim como evidência sobre as vias de transmissão que ligam as interfaces animais de produção - animais selvagens - ambiente. Pensa-se que a infeção por MAP possa resultar do contacto com material fecal contaminado ou o consumo de leite e colostro contaminados, havendo também evidência de transmissão intrauterina e, potencialmente, transmissão por via aérea. A contaminação ambiental pode também ter um papel importante na transmissão indireta, visto que o solo e a água são reservatórios de bactérias excretadas por animais infetados. Há evidência de que estas bactérias conseguem sobreviver no solo durante cerca de um ano, dependendo das condições climatéricas a que estão sujeitas, e cerca de 36 semanas em água.

A transmissão de MAP entre animais domésticos e selvagens pode ocorrer por diversas vias, dependendo dos hábitos alimentares de cada espécie. Os herbívoros poderão contrair a doença através do consumo de vegetação contaminada e os roedores através do consumo de ração contaminada. Já os carnívoros parecem contrair a doença indiretamente, através do consumo de presas infetadas. A transmissão entre animais selvagens e domésticos ocorre através do consumo de vegetação e ração contaminadas por herbívoros e roedores infetados. No entanto, segundo os estudos disponíveis, a excreção de MAP pelos animais selvagens não é significativa quando comparada com a dos animais de produção. Ainda assim, existe risco de introdução de MAP em manadas de bovinos não infetadas, através da contaminação ambiental.

Pensa-se que MAP possa ter potencial zoonótico, dadas as semelhanças entre a paratuberculose nos mamíferos e a doença de Crohn nos humanos. Existe evidência que MAP consegue resistir aos processos de tratamento de água para consumo humano e à pasteurização, pelo que a transmissão ao Homem pode ocorrer através do consumo de água, leite ou outros laticínios contaminados.

A paratuberculose é considerada endémica na maioria dos países da Europa, incluindo Portugal, onde há a necessidade de implementação de planos eficazes de vigilância e controlo. A infeção por MAP foi já confirmada em ruminantes domésticos de várias regiões do país, registando-se uma elevada prevalência ao nível das manadas. A infeção de animais selvagens foi também esporadicamente confirmada por estudos observacionais focados em diferentes espécies de mamíferos; no entanto, apenas um estudo recente focado em carnívoros abordou os potenciais fatores de risco.

Assim, este trabalho teve como objetivo avaliar que variáveis podem promover a infeção por MAP entre animais de produção, animais selvagens e o ambiente usando como área de estudo uma exploração agro-silvo-pastoril, a Companhia das Lezírias (CL). Os objetivos incluíram estimar a prevalência de MAP na comunidade de mamíferos da CL através de trabalho de campo e abordagens moleculares; avaliar a distribuição espacial da ocorrência de MAP; testar, através de modelos lineares generalizados mistos

(GLMMs), que fatores poderão modelar a exposição dos mamíferos a este microrganismo, e realizar inferências espaciais do risco de ocorrência de MAP na área de estudo.

Recolheu-se 206 amostras de dejetos de 11 espécies de mamíferos, 80 amostras de solo, e 150 amostras de bovinos pertencentes a seis manadas. Usando a sequência de inserção IS*900* como *proxy*, detetou-se MAP em amostras de 10 espécies de mamíferos (*Erinaceus europaeus*, *Genetta genetta*, *Herpestes ichneumon*, *Lepus granatensis*, *Martes foina*, *Meles meles*, *Mustela nivalis*, *O. cuniculus*, *Sus scrofa* e *Vulpes vulpes*), com uma prevalência global de 22%; em todas as manadas de bovinos, com uma prevalência individual de 54%; e em amostras de solo com 44% de prevalência. Este estudo é o primeiro a detetar MAP em ouriço-cacheiro (*E. europaeus*) em Portugal. Devido ao número limitado de amostras por espécie, não foi possível estimar a prevalência de MAP na maioria das espécies. As espécies com maior número de amostras foram o coelho-bravo (*O. cuniculus*) e a raposa (*V. vulpes*), com 62 e 37 amostras analisadas, respetivamente. Apesar de não ser uma espécie ruminante, o coelho-bravo é considerado um dos mais importantes hospedeiros na propagação de MAP, uma vez que pode excretar elevados níveis destas bactérias nos seus dejetos, promovendo a infeção de animais domésticos que consomem vegetação contaminada por estes dejetos. Neste trabalho, obtivemos 19% de amostras positivas de coelho-bravo e 22% de raposa. A elevada prevalência de MAP registada nas amostras de coelho-bravo pode estar na origem da prevalência observada na raposa e nos restantes carnívoros analisados, visto que o coelho-bravo é uma das principais presas dos mesocarnívoros em Portugal.

A elevada prevalência de MAP observada nas amostras de bovinos e solo pode perpetuar a infeção de bovinos e representar um elevado risco de transmissão para os animais selvagens.

Contrariamente ao esperado, a análise por GLMM sugeriu que a deteção de MAP no solo não influencia a presença de MAP em dejetos de mamíferos, o que pode estar relacionado com o limitado número de amostras analisado. Por outro lado, dada a natureza não-invasiva deste estudo em animais selvagens, desconhece-se a viabilidade de MAP (imprescindível para a infeção) nas áreas onde a sua presença foi detetada em DNA ambiental, pelo que não se pode concluir, com os dados atualmente disponíveis, qual o estatuto de infeção das populações nas áreas contaminadas. A diversidade de animais selvagens apresentou uma influência positiva na deteção de MAP nos mamíferos, enquanto a sua abundância mostrou uma influência negativa. Algumas variáveis relacionadas com o uso do solo exercem diferentes influências em grupos de mamíferos específicos: a cobertura de floresta mista teve uma influência positiva na presença de MAP em dejetos de carnívoros, enquanto a cobertura de habitat com predominância de vegetação arbustiva teve uma influência positiva na presença de MAP em dejetos de coelho-bravo. Surpreendentemente, a prevalência de MAP em bovinos mostrou uma influência negativa na presença de MAP em dejetos de lagomorfos. Este resultado inesperado pode advir da menor densidade de coelho-bravo nas zonas mais usadas pelos bovinos ou da distribuição heterogénea das amostras analisadas.

A inferência espacial do risco de infeção por MAP nos mamíferos resultou em dois *hotspots*; no entanto, o modelo gerado a partir das variáveis significativas mostrou suporte insuficiente (AUC = 0.661). Concluímos assim que as variáveis consideradas na construção do modelo não foram suficientes para prever de forma exata o risco de infeção por MAP em mamíferos. Estudos adicionais serão necessários para realizar esta previsão na nossa área de estudo e, futuramente, a nível nacional. Seria benéfico aumentar o número de espécimes para cada espécie já amostrada, aumentar o leque de espécies, e considerar novas variáveis bióticas e abióticas. Também o aumento do número de amostras ambientais (solo, vegetação e cursos de água) e da sua distribuição espacial poderá fornecer pistas importantes em estudos futuros. Para além de uma abordagem molecular baseada na análise de DNA ambiental, sugere-se uma abordagem cultural, por forma a demonstrar a viabilidade de MAP nas matrizes analisadas, apesar dos constrangimentos metodológicos para a cultura e isolamento desta bactéria fastidiosa.

Este estudo permitiu um avanço do conhecimento sobre a distribuição de MAP na comunidade de mamíferos na Companhia das Lezírias, proporcionando oportunidades de intervenção, nomeadamente

medidas de gestão de bovino infetados, aumentando também o nível de informação disponível à escala nacional. Acresce a confirmação da presença de MAP no solo, que poderá constituir uma potencial fonte de infeção de animais domésticos e selvagens.

Palavras-chave
Interfaces animais de produção - animais selvagens – ambiente; mamíferos; *Mycobacterium avium* subsp. paratuberculosis; transmissão

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ABBREVIATIONS AND ACRONYMS

ΔAICc - difference between the model's AICc and the smallest AICc value
AICc – Akaike Information Criteria, corrected for small samples
AUC – Area Under the Curve
bp – base pairs
CL – Companhia das Lezírias S.A.
Cq – Threshold Cycle
EE – *Erinaceus europaeus*
ELISA – Enzyme-Linked Immunosorbent Assay
GG – *Genetta genetta*
GLMs – Generalized Linear Models
GLMMs – Generalized Linear Mixed Models
HI – *Herpestes ichneumon*
LG – *Lepus granatensis*
LL – *Lutra lutra*
MAC – *Mycobacterium avium* complex
MAP – *Mycobacterium avium* subspecies *paratuberculosis*
MF – *Martes foina*
MM – *Meles meles*
MN – *Mustela nivalis*
OC – *Oryctolagus cuniculus*
OIE – World Organization for Animal Health
PCR – Polymerase Chain Reaction
SS – *Sus scrofa*
VIFs – Variation Inflation Factors
VV – *Vulpes vulpes*
WHO – World Health Organization

1. INTRODUCTION

Animal infectious diseases have high socio-economic and ecological impacts, posing serious threats to animal health and welfare, farmer livelihoods, livestock industries, food safety, public health, biodiversity and wildlife conservation. Although infectious diseases have rarely been cited as the main cause of species extinctions[1], there is evidence that they can cause population declines (e.g. myxomatosis in Iberian hares[2]), and can also interact with other factors that greatly contribute to these declines (e.g. interaction of diseases with climate change in amphibians[3])[4].

Intensification of human-dominated landscapes increases the emergence of infectious diseases due to the greater contact between humans, wildlife and livestock, which promotes intraspecific and interspecific pathogen transmission[5]. One such disease is paratuberculosis, or Johne's disease, a chronic infection caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). It is characterized by inflammation of the small intestine, with thickened and edematous walls, swollen and edematous mesenteric lymph nodes, and lesions in the liver and hepatic lymph nodes[6]. Infected animals suffer from diarrhea, submandibular edema, weight loss, malnutrition, anemia, lethargy, and even death[6].

MAP is included in the *Mycobacterium avium* complex (MAC), a group of environmental mycobacteria found in numerous natural and anthropogenic habitats[7]. MAC bacteria are phenotypically diverse, opportunistic pathogens of animals and humans, with a broad host range[7]. They can grow or survive in the environment, without losing infection ability, in wide temperature and pH ranges, showing rapid adaptation to new substrates[7]. Some characteristics distinguish MAP from other MAC species, namely its extremely slow growth and inability to produce mycobactin[8]. Isolation of MAP through culture, the gold standard, is highly hampered by its slow growth. Therefore, adoption of other methods, like molecular approaches[9,10], has become increasingly common for the diagnosis of infection. MAP can be detected in tissues, milk or feces by amplification of the insertion sequence IS*900* or the F57 sequence[10,11]. The IS*900* is defined as a 1,451 bp multicopy element inserted into 15 to 20 conserved *loci* in the MAP genome, depending on the strain[12], and has been the marker of choice for most molecular assays. However, according to Englund and collaborators (2002)[13], there is evidence of IS*900*-like sequences in other *Mycobacterium* species that may also be detected through polymerase chain reaction (PCR) and result in false positives for MAP. In contrast, the F57 sequence is highly specific for MAP detection, but it is present only in one copy, and so analytical methodologies based on F57 amplification have reduced sensitivity when compared to IS*900*[10].

MAP seems to primarily affect domestic ruminants (e.g. sheep, goats and cattle), although infection in wild ruminants is also well documented[14,15]. It has also been reported in monogastric domestic animals, such as pigs[16]. In non-ruminant wildlife, MAP was first detected in European rabbits in Scotland[17], and afterwards in a broad range of hosts, including birds[18] and diverse mammal *taxa*, such as lagomorphs[18], carnivores[18], rodents[18], ungulates[19] and primates[20]. MAP has also been isolated in insects[21] and earthworms[22].

MAP infection can happen through ingestion of contaminated fecal material or contaminated colostrum or milk, but also by intrauterine transmission and, potentially, via aerogenic transmission[23]. Environmental contamination can play a major role in transmission, since soil and water are reservoirs of bacteria excreted by infected animals. MAP has been known to survive in the environment for up to one year[24], and up to 36 weeks in water[25]. Animals with less than six months of age are more susceptible to infection[26], but they can remain asymptomatic for two to five years[27]. Asymptomatic undetected animals significantly contribute to sustained MAP transmission, since they are able to shed bacteria through milk and feces[26], infecting other animals and further contaminating the surrounding environment. Clinical disease is more common in livestock, although similar clinical signs have been found in wild ruminants[15] and severely infected rabbits[28]. In other non-ruminant species, such as carnivores, birds and rodents, only mild lesions have been reported[18]. Lack of histological lesions in

infected wild animals may be associated with an early phase of infection, the animal species or the MAP strain[29].

Mokresh and Butler (1990)[30], and later Greig and collaborators (1999)[31], showed that a MAP strain from cattle can infect wild rabbits, suggesting that transmission between livestock and wildlife could occur. Many biotic and abiotic factors may contribute to the transmission of MAP to wildlife, such as microhabitat, water and soil conditions, herd size, husbandry practices, species interactions, and grooming and feeding habits[31]. Conversely, Beard and collaborators (2001)[32] have also demonstrated experimentally that a strain from a wild rabbit can infect cattle, which suggests that transmission from wildlife to livestock also occurs.

Livestock to wildlife transmission may occur in two different ways: through the ingestion of contaminated vegetation by herbivore species, or through the ingestion of contaminated livestock feed by rodents[33], as high amounts of bacteria are excreted in livestock feces[34,35]. Likewise, wildlife to livestock transmission might occur through the ingestion of pasture or feed contaminated with rabbit or rodent feces, respectively, which cattle do not appear to avoid[36,37]. On the other hand, carnivores may be infected indirectly through predation of infected prey or carcasses[33]. This last hypothesis is supported by the higher prevalence of infection observed in predators than in their prey[33]. Carnivores are not considered epidemiologically important for MAP transmission to livestock, since domestic animals seem to avoid carnivore feces[38]. In any case, since carnivores cover large distances, they could spill over bacteria into the environment and play a role in the spatial spread of MAP[33]. MAP excretion by wildlife is not significant compared to livestock; nevertheless, they can still pose a risk to paratuberculosis-free herds by introducing the pathogen in the environment[29].

Research about MAP's zoonotic potential is ongoing, since similarities between paratuberculosis in mammals and Crohn's disease in humans have been observed[9]. Studies suggest that MAP survives engineered water systems and treatments[7], as well as pasteurisation[13], which poses a significant risk of transmission to humans through the consumption of contaminated water and dairy products.

In most countries in Europe, paratuberculosis is considered endemic, including in Portugal[39], where an official surveillance and control plan is lacking. However, countries like Sweden have maintained a paratuberculosis-free status, using rigorous control programs[39].

In Portugal, MAP herd prevalence in small domestic ruminants has been estimated between 6-18% in Alentejo[40], 27% in Estremadura[41] and 66.7% in Trás-os-Montes e Alto Douro[42] regions. In cattle, herd prevalence has been estimated between 13-25% in Alentejo[40] and up to 45.9% in the Northern region[43]. In wildlife, MAP has been reported in red deer (*Cervus elaphus*, 4.2%)[44], wild boar (*Sus scrofa*, 28.9%)[45], European rabbit (*O. cuniculus*, 7.9%)[46], and many carnivore species, such as Egyptian mongoose (*Herpestes ichneumon*), red fox (*Vulpes vulpes*), stone marten (*Martes foina*), common genet (*Genetta genetta*), Eurasian otter (*Lutra lutra*) and European badger (*Meles meles*), with prevalences varying from 7.43% to 27%[11,47].

Altogether, studies so far show that prevention and control programs applied to livestock are necessary in order to prevent intraspecific transmission and the underlying health effects and economic losses, but also to minimize transmission between livestock and wildlife, breaking the infection cycle. Rapid detection of MAP and application of real-time control measures are vital to decrease the impact of paratuberculosis on the economy sustained by livestock production, but also on public health and wildlife. Systematic surveillance and monitoring provide data useful to identify and quantify explanatory variables for observed MAP outbreaks, offering opportunities for control. For example, currently unanswered questions include if climate, land cover, proximity to human settlements, species occurrence and distribution, or animal husbandry, are particularly linked to observed spatial patterns of MAP occurrence. And if so, could these variables be used to predict MAP prevalence and risk in unobserved locations?

Thus, this thesis aimed to study which variables promote MAP infection between livestock, wildlife and the environment using a Mediterranean agro-forestry farmstead, Companhia das Lezírias (CL), as a model. Although focused in agriculture, forestry and cattle production, wildlife conservation, including habitat restoration plans currently in course[48], is also a priority, making CL a diverse area in terms of land use and wildlife. Thus, CL may help understand potential livestock-wildlife-environment interactions. The specific objectives of this thesis included: (1) estimating MAP prevalence in the mammal community by means of field work and wet lab molecular approaches; (2) assessing the spatial distribution of MAP infection in the sampled mammal community; (3) test which factors modulate wildlife exposure to MAP; and (4) predict MAP infection risk within the study area.

2. METHODS

2.1. Study area

Companhia das Lezírias (CL) is the largest agro-forestry farmstead in Portugal, located in Santarém. The farmstead is divided into two areas: the Lezíria (8000 ha), located between the Tagus and Sorraia rivers, characterized by rich alluvium soils; and the Charneca do Infantado (10000 ha), where this work took place, characterized by poor sandy soils, with poor drainage and a predominance of shrubby vegetation[49]. The study area is characterized by a Mediterranean climate, with dry and warm summers, and wet and cold winters.

Land use in CL includes pasture for cattle production; forest, mainly for cork, wood and pinecone production; and agriculture fields for rice, corn, wine and olive oil production[48] (Figure 2.1). Riparian habitats constitute one of the few natural, or less disturbed, habitats in the area, with most watercourses being small and intermittent[50]. CL focuses on a sustainable land management, prioritizing extensive husbandry systems for cattle production, with natural pastures included in a traditional landscape, the *montado* (a type of grassland associated with *Quercus* trees).

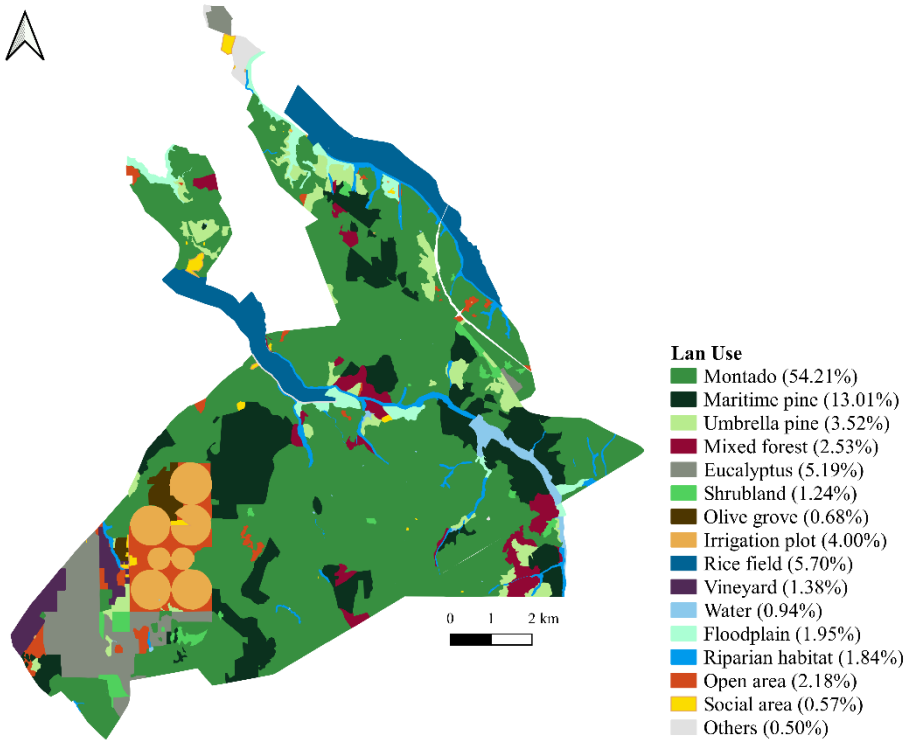

Figure 2.1: Land use in Charneca do Infantado in 2020. Information provided by CL.

Around 53% of CL's territory is included in the Natura 2000 Network, included in the Special Protection Zone and Site of Community Interest of the Tagus Estuary, with the codes PTZPE0010 and PTCON0009, respectively[48]. CL's mammal community is composed of 21 non-flying species from five orders (Cetartiodactyla: 1 sp., Carnivora: 9 spp., Eulipotyphla: 4 spp., Lagomorpha: 2 spp. and Rodentia: 5 spp.) (Supplementary table 1)[51]. Among these, only a few have a conservation status of concern in Portugal: *Oryctolagus cuniculus* (near-threatened), *Microtus cabrerae* (vulnerable), and *Mustela putorius* (data deficient)[52]. *Felis silvestris* is considered vulnerable[52]; however, this species was not confirmed in our study area, even though the presence of first-generation hybrids was confirmed by molecular assays. Furthermore, recently, two new species were detected in our study area, *Dama dama* and *Sciurus vulgaris*.

2.2. Field sampling

2.2.1. Wild species and soil sampling

The study area was divided into a 1x1 km grid (approximately 105 cells), from which 80 cells were randomly selected for sampling: 40 cells were sampled between November and December of 2020 and the other 40 between March and May of 2021 (Figure 2.2).

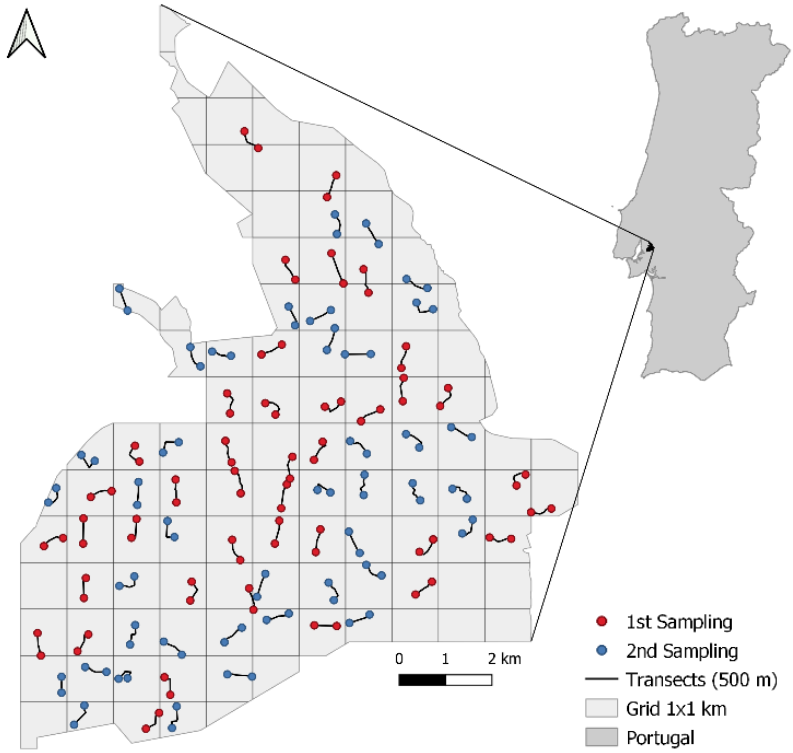

Figure 2.2: Study area with sampled transects. Charneca do Infantado, in CL, with the sampled linear transects during two field campaigns in late 2020 (n = 40) and early-to-mid 2021 (n = 40). Colored dots represent the start and finish points of each transect.

Each cell included a 500 m linear transect (Figure 2.2) and a second path following a rough zigzag pattern, around 50 m to the side of the main path, for a total of 1.5 km per transect (Figure 2.3). In each transect, all mammal feces that could be spotted were collected into individual sterile zipped plastic bags, georeferenced and identified at the species level based on morphological criteria, considering its size, shape and placement[53]. *O. cuniculus* latrines were considered as a single sample, since individual feces cannot be identified, similarly to *M. meles,* where fecal material collected from each latrine was merged into a single sample.

Additionally, in each transect, one soil sample was collected randomly into a 50 mL sterile conical tube and georeferenced. Whenever possible, soil samples were collected near water (e.g. puddles, ponds).

All samples were stored at -20ºC until needed for laboratory analyses, in agreement with the World Organization for Animal Health (OIE) and the World Health Organization (WHO) guidelines.

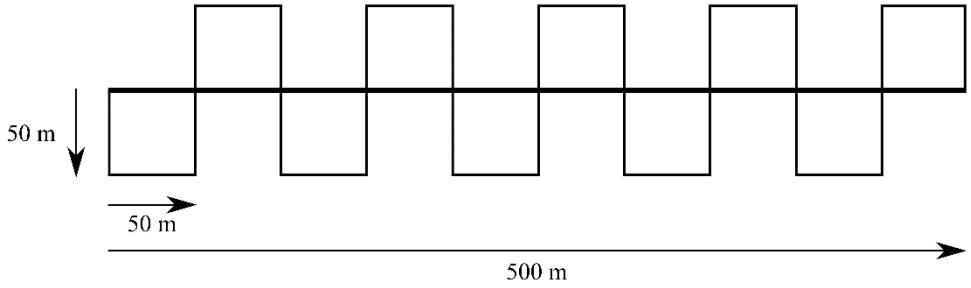

Figure 2.3: Transect scheme. Linear transect of 500 m, including another transect following a zigzag pattern of 50 m for each side of the main path.

2.2.2. Cattle sampling

At the time of the study, CL owned 1421 cattle heads from two native breeds (*Preta* and *Mertolenga*), two exotic breeds (*Charolesa* and *Limousine*) and a crossbreed between native and exotic breeds (*Cruzada*). The animals were divided into nine herds (*Charolesa* (n = 37), *Limousine* (n = 52), *Mertolenga A* (n = 213), *Mertolenga B* (n = 190), *Preta C* (n = 112), *Preta D* (n = 158), *Preta E* (n = 213), *Cruzada F* (n = 169) and *Cruzada G* (n = 165)) composed by reproductive females and their calves that rotate pastures periodically considering the grazing pressure (Figure 2.4). Although the herds are usually separated, they are frequently in adjacent pastures and some areas can be used by more than one herd at different times[54]. Males are strategically introduced in herds for reproduction purposes and stay through all the female's gestation period[55]. After birth, calves stay with their mother until weaning and are then selected for reproduction purposes (in which case they stay in the herd) or meat production (in which case they are transferred to a semi-intensive system)[55]. Every year, all herds are moved from Charneca do Infantado to Lezíria, where they stay during Autumn/Winter and Spring/Summer, respectively[48].

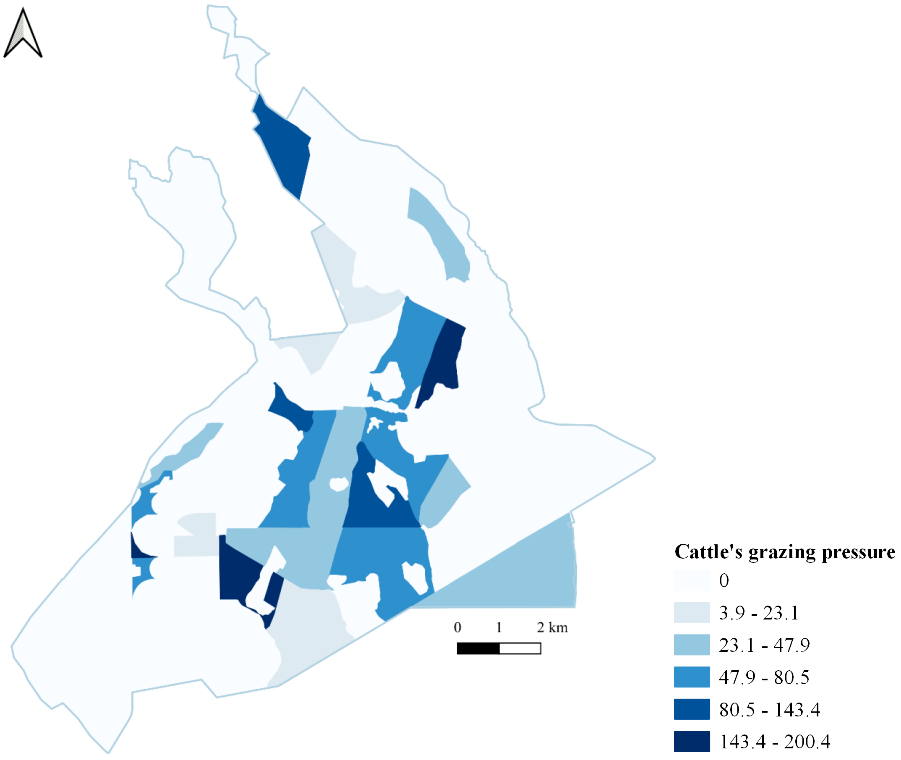

Figure 2.4: Grazing pressure in Charneca do Infantado in 2020-2021, calculated as the number of individuals per hectare multiplied by the number of days. Information provided by CL.

Between May and July of 2021, 25 fecal swab samples were collected from each of six cattle herds (*Limousine*, *Mertolenga B*, *Preta C*, *Preta D*, *Preta E* and *Cruzada F*), summing 150 samples (representing 10% of overall cattle population size). Only females with more than 2 years of age were sampled, chosen randomly within each herd. The samples were collected during intradermal tuberculin testing, which limited sampling to six out the nine herds during our study period. Swabs were transiently stored in a Styrofoam box with ice and kept at -20 ºC in the lab until further processing.

2.3. Laboratory analyses

Genomic DNA was extracted from 500 mg of soil or feces using the NZY Soil gDNA Isolation kit (NZYTech); and from fecal swabs in 600 µL of Phosphate Buffer Saline (PBS) using the NZY Tissue gDNA Isolation kit (NZYTech), following the manufacturer's instructions. Genomic DNA suspensions were stored at -20°C until needed for PCR assays.

*2.3.1. Nested real-time PCR targeting IS*900

Presence of MAP was evaluated using a nested real-time PCR targeting the insertion sequence IS*900*[11]. This approach shows higher sensitivity compared to a single-step real-time PCR[10,27]. The first amplification step consists of a standard PCR targeting a 224 bp sequence, located between positions 204 and 427 of the IS*900* sequence[11]. The reaction was carried out in a final volume of 25 µl containing 1x NZYTaq Colourless Master Mix (NZYTech), 20 µM of each IS*900*-targeted primer (IS*900*-EXT-FW and IS*900*-EXT-RV) (Table 2.1), DNase-free water, and 5 µl of the extracted DNA. The amplification was performed in an UNO II Thermal Cycler (Biometra), with the following steps: denaturation step at 95°C for 3 min, and amplification during 35 cycles including denaturation (95°C for 45 s), annealing (55ºC for 30 s) and extension (72°C for 90 s), and a final step of extension (72ºC for 10 min)[56]. All amplified products were stored at 4°C until further use. The second amplification step consists of a real-time PCR targeting an inner IS*900* sequence with 66 bp[11], using the previous amplification product as template. The reaction was carried out in a final volume of 20 µl containing 1x NZY qPCR Probe Master Mix (NZYTech), 20 µM of each IS*900*-targeted primer (IS*900*-INT-FW and IS*900*-INT-RV) and probe (TET and BHQ1) (Table 2.1), DNAse-free water and 5 µl of the product from the previous PCR reaction. The amplification was performed in a CFX96 Real-Time Thermal Cycler (BioRad), with the following steps: denaturation step at 95°C for 2 min, and amplification during 45 cycles including denaturation (95°C for 5 s), annealing and extension (60ºC for 10 s)[56]. Samples were considered positive when the threshold cycle (Cq) was below 35; for samples that crossed the threshold line after 35, analysis in 2% (w/v) agarose gel were performed to confirm amplification of fragments with expected size.

In addition to samples, a positive control (DNA extracted from MAP ATCC 19698) and a no-template control (water) were included in all PCR batches. The amplified products from both PCR reactions were regularly visualized in a 2% (w/v) agarose gel, yielding fragments of 224 bp and 66 bp, respectively. Precautions were taken during all stages to avoid nucleic acid carry-over. Only experiments wherein negative and positive controls performed correctly were considered.

2.3.2. Real-time PCR targeting F57

Another real-time PCR targeting the specific, single copy F57 sequence (80 bp) of MAP was performed for all IS*900*-positive samples[11]. The reaction was carried out in a final volume of 20 µl containing 1x NZY qPCR Probe Master Mix (NZYTech), 20 µM of each F57-targeted primer (F57-FW and F57-RV) and probe (FAM and TAMRA) (Table 2.1), DNase-free water and 5 µl of the extracted DNA. The amplification was performed in a CFX96 Real-Time Thermal Cycler (BioRad), with the

following steps: denaturation step at 95°C for 10 min, and amplification during 45 cycles including denaturation (95°C for 15 s), annealing, and extension (60°C for 1 min)[10]. In addition to samples, a positive control (DNA extracted from MAP ATCC 19698) and a no-template control (water) were included in all PCR batches. Only experiments wherein negative and positive controls performed correctly were considered.

Table 2.1: Primers and probes used in the nested real-time PCR targeting IS*900* and in the real-time PCR targeting F57[10,11,27].

Target gene	Primers and Probes	Sequence (5' → 3')	Product
IS*900*	IS*900*-EXT-FW	TGATCTGGACAATGACGGTTACGGA	224 bp
	IS*900*-EXT-RV	GGCGTTGAGGTCGATCGCCCACGTGAC	
	IS*900*-INT-FW	CCGGTAAGGCCGACCATT	66 bp
	IS*900*-INT-RV	ACCCGCTGCGAGAGCA	
	IS*900* probe	TET – CATGGTTATTAACGACGACGCGCAGC – BHQ1	
F57	F57-FW	AACTAAGCGGATCGACAATTC	80 bp
	F57-RV	TGGTGTACCGAATGTTGTTG	
	F57 probe	FAM – TGCAACTCGAACACACCTGGGA – TAMRA	

*2.3.3. Sequencing analyses to confirm the specificity of IS*900 *amplicons*

To confirm the specific amplification of IS*900* rather than IS*900*-like sequences, four amplicons were chosen for sequencing: two from wildlife, one from cattle and one from soil. The four amplicons that resulted from the first PCR step (224 bp) in the nested approach were extracted from 2% (w/v) agarose gel, purified using the NZYGelpure kit (NZYTech) (following the standard protocol) and commercially sequenced (Eurofins Genomics, Germany)[11]. The obtained sequences were then compared with sequences from public databases (BLAST, blast.ncbi.nlm.nih.gov/Blast.cgi) in order to find regions of similarity corresponding to IS*900*. Then, using MEGA-X 10.2.6, the sequences were aligned with eleven known IS*900* sequences (accession numbers AJ250023.1, AJ250018.1, AJ250016.1, AJ250017.1, AJ250015.1, AJ250021.1, AJ250022.1, S74401.1, AF416985.1, X16293.1 and HM015765.1) and two IS*900*-like sequences (*Mycobacterium* sp. 2333, accession number AF455252.1 and *Mycobacterium porcinum*, accession number EU126150.1) that were retrieved from NCBI nucleotide database[11] (accessed in August 2021).

2.4. Statistical analyses

Wildlife's sample spatial distribution was investigated using a linear regression between the number of samples of each species and the number of transects in which they were collected, to assess possible sampling biases.

Wilcoxon-Mann-Whitney tests were performed to test for differences in presence/absence of MAP between sampling campaigns in wildlife and between cattle herds. They were also performed to test for differences in Cq values that resulted from the second step of the nested real-time PCR targeting IS*900*. The assumptions were verified using the Shapiro-Wilk test for normality and the Bartlett test for homogeneity. Statistical significance was accepted for *p*-values lower than 0.05.

Data visualization was performed with the "ggplot2" package[57] in R (version 4.1.2) and maps were built using QGIS 3.12.2.

2.4.1. Factors influencing MAP-infection in wildlife

To study which factors could influence MAP infection risk at the livestock-wildlife-environment interfaces, using detection of IS*900* as proxy, we formulated five hypotheses respectively related to climatic conditions, land use, host abundance, aggregation points and environmental MAP contamination (Table 2).

All the hypotheses were studied individually and for four different datasets: data from all species with more than 10 samples (A); data from all carnivore species (B); data from all lagomorph species (C); data from *Oryctolagus cuniculus* (D). For datasets A and D, we considered a buffer of 100 m around each sample, and for B and C we considered the mean core area of the species as the buffer – 285 m radius for carnivores[58,59] and 87.5 m radius for lagomorphs[60,61].

Climatic conditions were calculated for each sample as the mean value of the seven days prior to sample collection. Land use variables were calculated for each sample location as a proportion of cover inside the corresponding buffer. For host variables, wildlife's abundance was calculated for each transect as the Kilometric Abundance Index (number of samples collected per kilometer); wildlife diversity was calculated for each transect with the Shannon-Wiener index using R (version 4.1.2) with the "vegan" package[62]; and cattle abundance was calculated for each sample as an index, multiplying the grazing pressure value of each plot by the percentage of the buffer area it covers. For aggregation variables, distances were calculated for each sample to the nearest point of water, wildlife feeders and humanized areas. Finally, for contamination variables, MAP contamination in soil was considered a binary variable based on presence/absence of IS*900* amplification in each transect's sample; and MAP in cattle was calculated for each sample as an index, multiplying the MAP prevalence of each plot by the percentage of the buffer area it covers.

Prior to the modeling procedure, spatial autocorrelation and variable multicollinearity was checked using Moran's I index[63] and variance inflation factors (VIFs)[64], respectively. Variables with a VIF value of 5 or above were excluded one at a time, that is, the variable with the higher value was excluded and VIFs were then recalculated until all variables had values below 5. All the continuous variables were then standardized to fit a scale between 0 and 1. To test which factors modulate presence/absence of MAP in wild mammals, Generalized Linear Mixed Models (GLMMs) with a binomial distribution were used, using the species as random effect, for datasets A, B and C; and Generalized Linear Models (GLMs), also with a binomial distribution, were used for dataset D. First, several models were built with every possible variable combination within the hypothesis. Then, all models were ranked according with their AICc (Akaike Information Criteria, corrected for small samples) and ΔAICc (difference between the model's AICc and the smallest AICc value), and those with a $\Delta AICc < 2$[65] were selected as best models. If more than one model was selected in the previous step, a model averaging procedure was implemented, and the variables' coefficients was estimated. Finally, the Area Under the Curve (AUC) was calculated to validate the best model under each hypothesis. Usually, AUC values of 0.5–0.7 suggest low accuracy, values of 0.7–0.9 suggest useful applications and values higher than 0.9 suggest high accuracy[66]. All analyses were performed using R (version 4.1.2) with the "scales" package for variable rescaling[67], the "lme4" package for model fitting[68], the "MuMIn" package for model averaging[69] and the "pROC" package[70] for AUC calculation. Data visualization was performed with the "ggplot2" package[57].

Table 2.2: Hypotheses studied. Description of the hypothesis explored to identify which variables promote MAP infection at the livestock-wildlife-environment interfaces in CL study area.

Hypothesis	Rationale	Variables (measure unit)	Variable Code
H1 Climatic conditions	Climatic conditions may influence permanence of MAP in the environment	• Temperature (ºC) • Relative humidity (%) • Rainfall (mm)	• temp • hum • rain
H2 Land use	Habitat use specificities by the different mammal species may influence transmission	• *Montado* cover (%) • Maritime pine cover (%) • Umbrella pine cover (%) • Mixed forest cover (%) • Shrubland cover (%) • Riparian habitat cover (%) • Olive grove cover (%) • Open habitat cover (%) • Eucalyptus cover (%) • Floodplain cover (%) • Rice field cover (%) • Irrigation plot cover (%)	• montado • m_pine • u_pine • mixed • shrub • rip • olive • open • euc • flood • rice • pivot
H3 Host abundance	Host diversity and abundance may influence MAP transmission	• Wildlife abundance • Wildlife diversity • Cattle abundance	• ika • diversity • cattle
H4 Aggregation points	Potential attraction points for animals feeding and drinking may increase interspecies transmission	• Distance to water (m) • Distance to rabbit feeders (m) • Distance to humanized areas (m)	• water • feeders • hum_areas
H5 Environmental MAP contamination	Environmental contamination may be an important route of transmission, since MAP can survive up to 1 year in soil	• MAP presence/absence in soil (binary) • MAP prevalence in cattle	• map_soil • map_cattle

2.4.2. Infection risk assessment

A new model was built for all species data using the statistically significant variables that resulted from the previous models. Here, statistical significance was accepted for *p*-values lower than 0.1. A GLMM with the species as random effect and binomial distribution was built as previously explained. The resulting averaged model was then used to predict the probability of infection in the study area, using the logit equation[71] (1), and mapped in QGIS 3.12.2.

$$\pi = \frac{1}{1 + e^{-(\beta_0 + \beta_1 x_1 + \cdots + \beta_k x_k)}} \quad , \tag{1}$$

where β_0 is the intercept's estimate value, β_k is the estimate value of the variable k, and x_k is the normalized raster of variable k.

The variables had to undergo prior preparation in QGIS. First, all variables of interest were mapped and converted into separate raster files. Since this data consists of points, the raster distance was calculated with the proximity algorithm, generating a map with the distance from the center of each pixel to the center of the nearest pixel identified as a target pixel (those in the source raster). Each raster was then normalized, using the equation (2), in raster calculator.

$$1 - \frac{1}{1 + 0.001 y_k} \quad , \tag{2}$$

where y_k is the raster distance of the variable k.

The normalized raster variables were used to calculate the logit equation (1) in raster calculator, generating a map of probability of MAP infection.

3. RESULTS

3.1. Wildlife sample diversity and distribution

Field sampling resulted in the collection of 206 fecal samples from wild mammals belonging to 11 species (*Sus scrofa* (*n* = 16), *Genetta genetta* (*n* = 4), *Herpestes ichneumon* (*n* = 6), *Lutra lutra* (*n* = 4), *Martes foina* (*n* = 11), *Meles meles* (*n* = 25), *Mustela nivalis* (*n* = 11), *Vulpes vulpes* (*n* = 37), *Erinaceus europaeus* (*n* = 19), *Lepus granatensis* (*n* = 11) and *Oryctolagus cuniculus* (*n* = 62)) (Figure 3.1), that can be grouped in four orders, Artiodactyla, Carnivora, Eulipotyphla and Lagomorpha. *O. cuniculus* and *V. vulpes* were the most represented species (30% and 18%, respectively). No samples were collected in 21 (17%) of the 80 transects sampled.

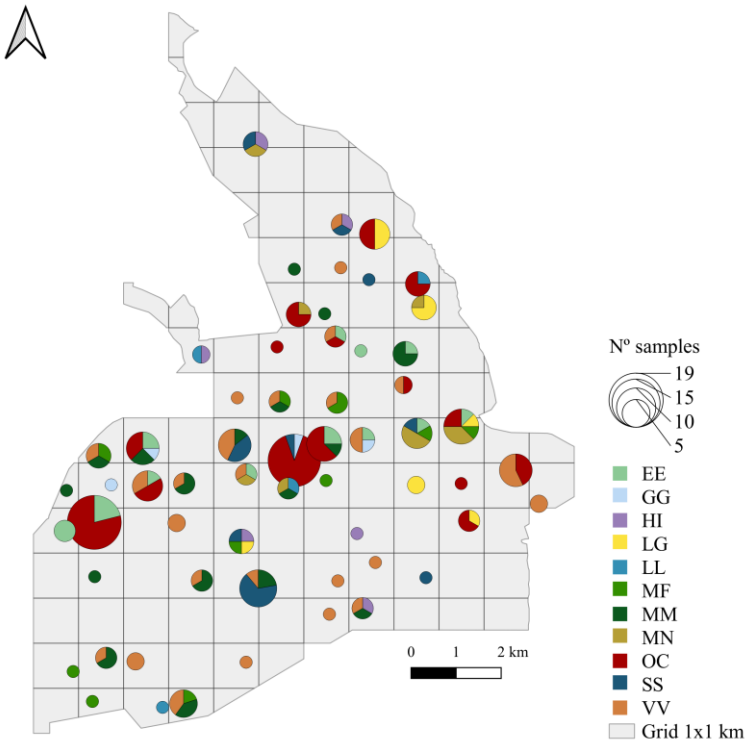

Figure 3.1: Distribution of the samples collected per transect. The size of the circles represents the number of samples analyzed. EE – *E. europaeus*, GG – *G. genetta*, HI – *H. ichneumon*, LG – *Lepus granatensis*, LL – *Lutra lutra*, MF – *Martes foina*, MM – *Meles meles*, MN – *Mustela nivalis*, OC – *Oryctolagus cuniculus*, SS – *Sus scrofa* and VV – *Vulpes vulpes*.

Results from linear regression showed a positive relationship between the number of samples from a particular species and their distribution ($r^2 = 0.521$), meaning that the species with higher relative abundance are better distributed throughout the study area. However, the r^2 value indicates that the regression model does not fit approximately 50% of the observations. In the resulting plot, *O. cuniculus* (the species with higher number of samples, *n* = 62) represents an outlier (Figure 3.2), contributing for the observed low r^2 value. Thus, we conclude that *O. cuniculus* is not well distributed, and so results from its data should be carefully discussed.

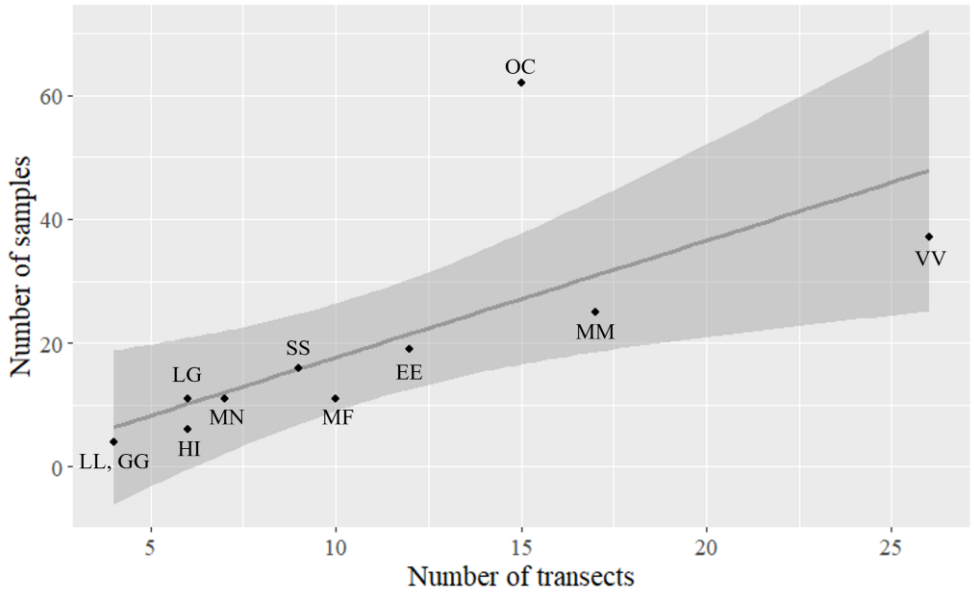

Figure 3.2: Linear regression between the number of wildlife samples from each species and the number of transects in which they were collected. Shaded areas represent 95% confidence intervals. EE – *E. europaeus*, GG – *G. genetta*, HI – *H. ichneumon*, LG – *Lepus granatensis*, LL – *Lutra lutra*, MF – *Martes foina*, MM – *Meles meles*, MN – *Mustela nivalis*, OC – *Oryctolagus cuniculus*, SS – *Sus scrofa* and VV – *Vulpes vulpes*.

*3.2. Molecular detection of MAP using IS*900 *as proxy*

Molecular detection of MAP-positive samples was evaluated by nested-real time PCR using only the IS*900* as proxy (Figure 3.3), since no results were obtained by the real-time PCR targeting the single-copy F57.

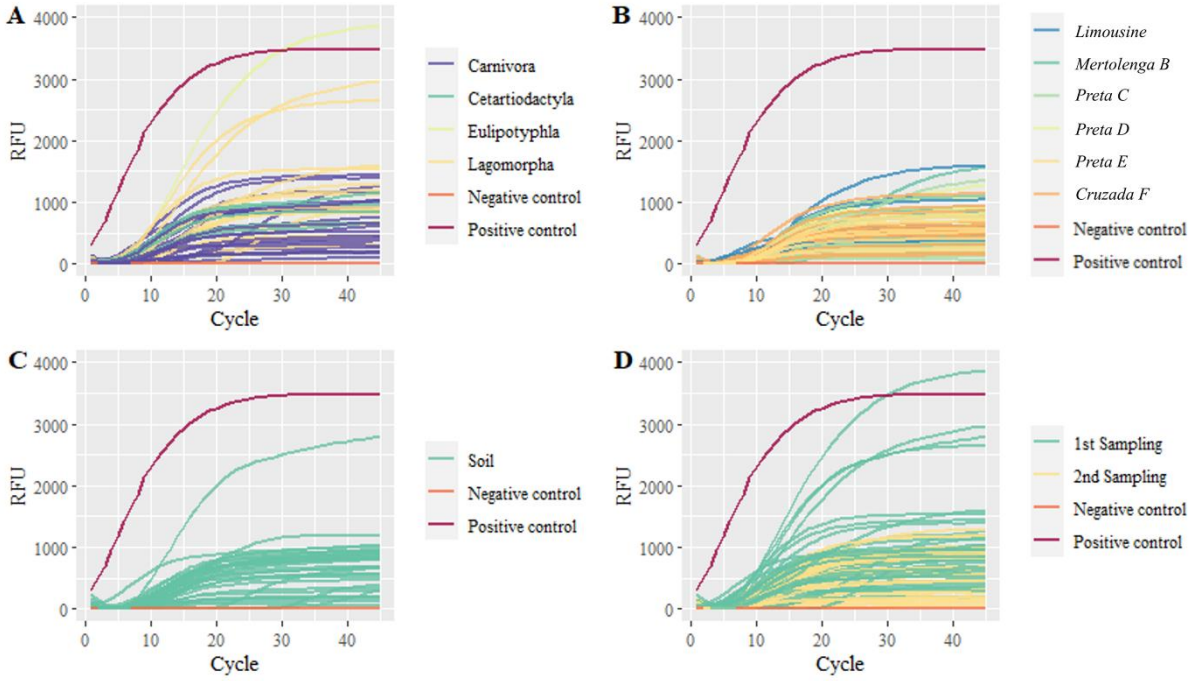

Figure 3.3: Real-time PCR amplification curves targeting IS*900*. Positive samples from: (A) each wildlife mammal group, (B) each cattle herd, and (C) soil. The real-time PCR amplification curves obtained during the first and second sampling campaign for wildlife and soil samples are depicted in D. RFU – Relative Fluorescence Units. The horizontal axes represent the amplification cycles.

The distribution of Cq values ranged up to 21.89 (mean = 7.17) in wildlife samples, up to 28.91 (mean = 8.74) in soil, and up to 16.40 (mean = 8.10) in cattle (Figure 3.4). Significant differences were observed between Cq values from cattle and wildlife (W = 1391.5, p = 0.028). Lower Cq values may reflect higher MAP concentration in the sample, better DNA extraction efficiency or better preservation of the sample[56].

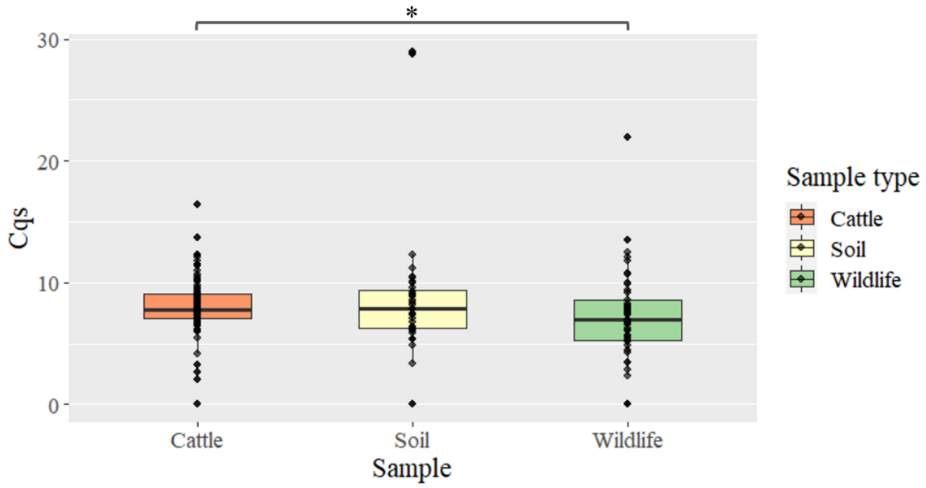

Figure 3.4: Distribution of the Cq values obtained in the real-time IS*900* PCR. Results from the second step of the nested real-time IS*900* PCR. The black dots represent each sample's Cq. The asterisk represents the significant difference between Cq values in cattle and wildlife (∗ – p-value < 0.05).

3.2.1. MAP in wildlife

Wildlife's seasonal data was taken as a whole for all analyses, since results obtained for all species, except *M. foina* (W = 28, p = 0.002), showed no significant differences between the two sampling campaigns (Supplementary figure 1). Overall, wildlife registered 22% (95% CI: 17-28) of MAP-positive samples. *G. genetta* was the species with the highest MAP prevalence (50%, 95% CI: 15-85), however the sample size is quite limited (n = 4) (Figure 3.5). Focusing on the two species with more samples analyzed, *O. cuniculus* (n = 62) registered 19% (95% CI: 11-31) of MAP-positive samples and *V. vulpes* (n = 37) had 22% (95% CI: 11-37) (Figure 3.5).

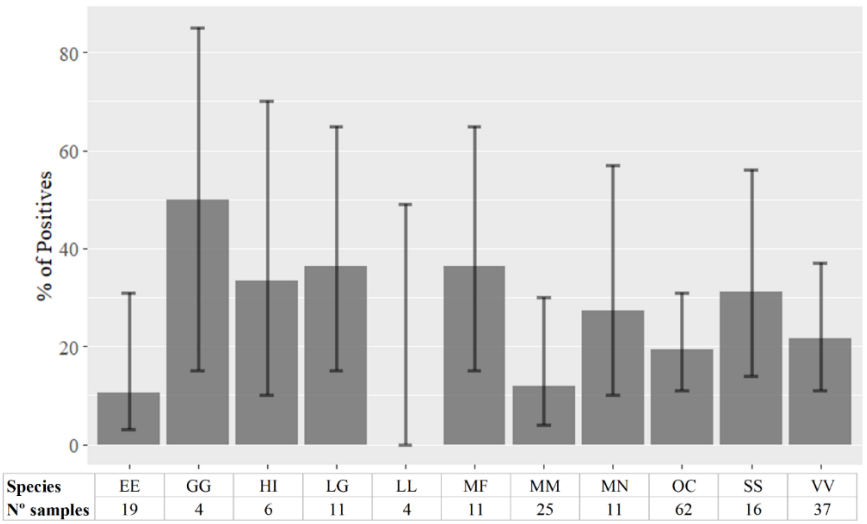

Figure 3.5: Prevalence of MAP in wildlife. Percentage of MAP-positive samples for each wildlife species for both sampling campaigns with the associated 95% confidence intervals. EE – *E. europaeus*, GG – *G. genetta*, HI – *H. ichneumon*, LG – *Lepus granatensis*, LL – *Lutra lutra*, MF – *Martes foina*, MM – *Meles meles*, MN – *Mustela nivalis*, OC – *Oryctolagus cuniculus*, SS – *Sus scrofa* and VV – *Vulpes vulpes*.

The number of samples analyzed is not equally distributed across the study area. Some transects had up to 19 samples, while most had fewer than 5 samples (mean = 2.59, sd = 3.41). Positive samples seem to be present throughout the study area with no apparent clustering (Figure 3.6).

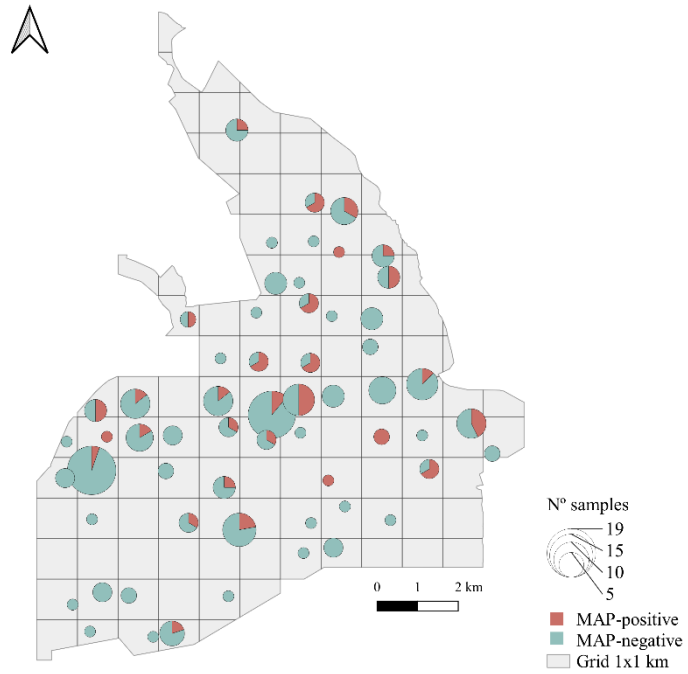

Figure 3.6: Distribution of wildlife positive and negative samples for MAP infection. The size of the circles represents the number of samples analyzed.

3.2.2. MAP in cattle

Overall, cattle registered 54% (95% CI: 46-62) of MAP-positive samples, and ranged between 32% and 76 % between herds (Figures 3.7 and 3.8). MAP-infection was observed in all herds sampled (100%).

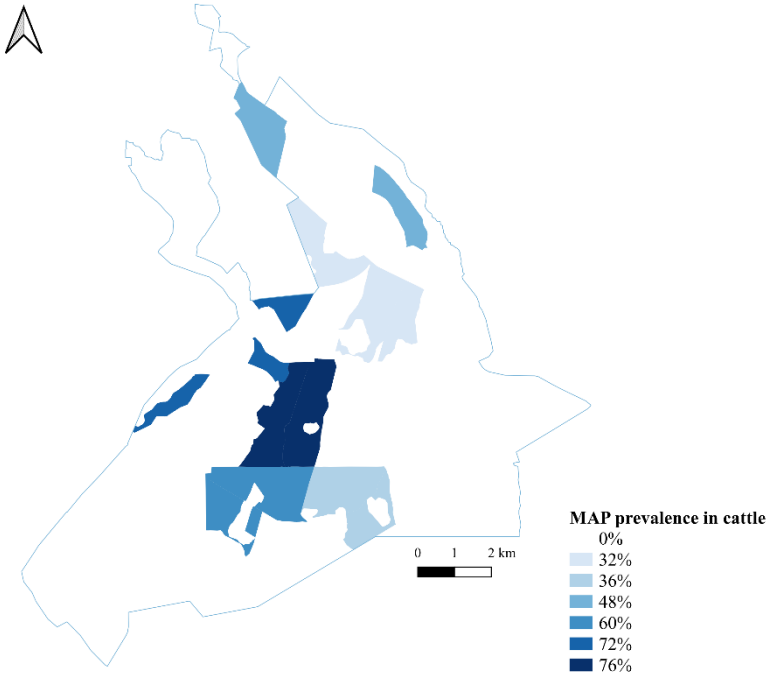

Figure 3.7: MAP prevalence in cattle in Charneca do Infantado, in 2020-2021. Prevalence of MAP was evaluated by amplification of the IS*900* sequence of 25 samples for each herd.

There are significant differences between most herds that registered the highest MAP prevalence, *Preta C* (76%; 95% CI: 57-89), *Limousine* (72%; 95% CI: 52-86) and *Cruzada F* (60%; 95% CI: 41-77), and the lowest, *Mertolenga B* (32%; 95% CI: 17-52), *Preta E* (36%; 95% CI: 20-55) and *Preta D* (48%; 95% CI: 30-67) (Figure 3.8).

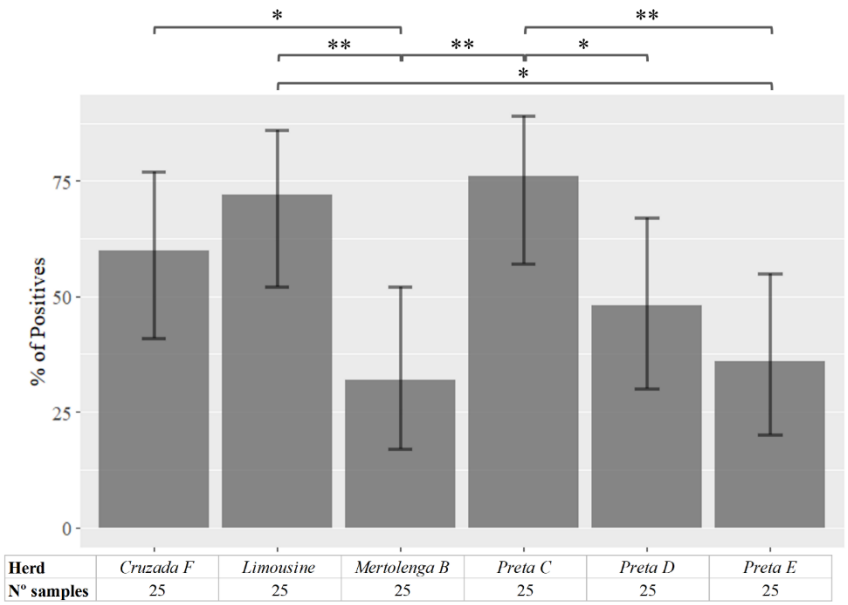

Figure 3.8: Prevalence of MAP in cattle. Percentage of positive samples for each cattle herd with the associated 95% confidence intervals. *n* – number of samples analyzed. The asterisks represent significant differences (∗ – *p*-value < 0.05; ∗∗ – *p*-value < 0.01).

3.2.3. MAP in soil

MAP was detected in 35 of the 80 transects sampled (Figure 3.9), with a prevalence of 44% (95% CI: 33-55). MAP in soil seems to be distributed across the study area with no apparent clustering (Figure 3.9).

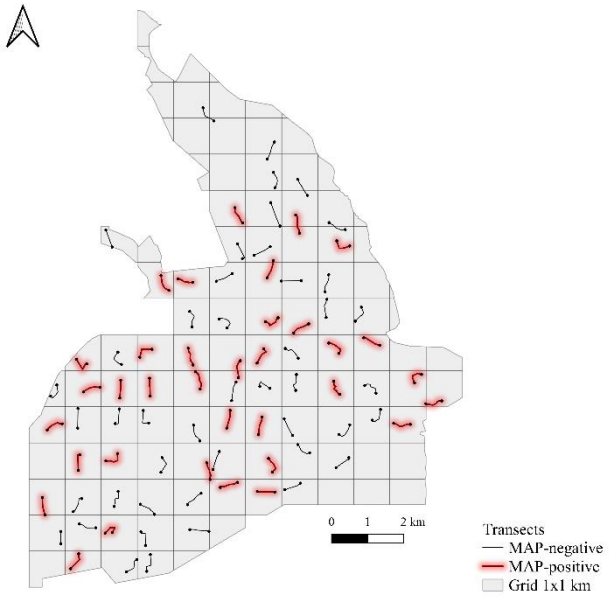

Figure 3.9: MAP in soil in Charneca do Infantado, in 2020-2021. Presence of MAP was evaluated by amplification of the IS*900* sequence in each transect's sample.

3.2.3. Confirmation of the specificity of IS900 amplicons

Electrophoretic analyses of IS*900* nested PCR products evidenced amplicons of the correct size. Specific MAP detection in wildlife, cattle and soil was confirmed by sequencing IS*900* amplicons generated by the first step of the nested approach using the first amplification primers. The obtained 224 bp amplicon sequences were aligned with IS*900* sequences from 11 MAP strains genomes deposited in GenBank and two IS*900*-like sequences from *Mycobacterium* sp. 2333 and *Mycobacterium porcinum*, showing 100% similarity with the MAP sequences from public datasets. The specificity of the IS*900* amplicons was then confirmed by the point mutation that distinguishes IS*900*-like from MAP IS*900* (Figure 3.10).

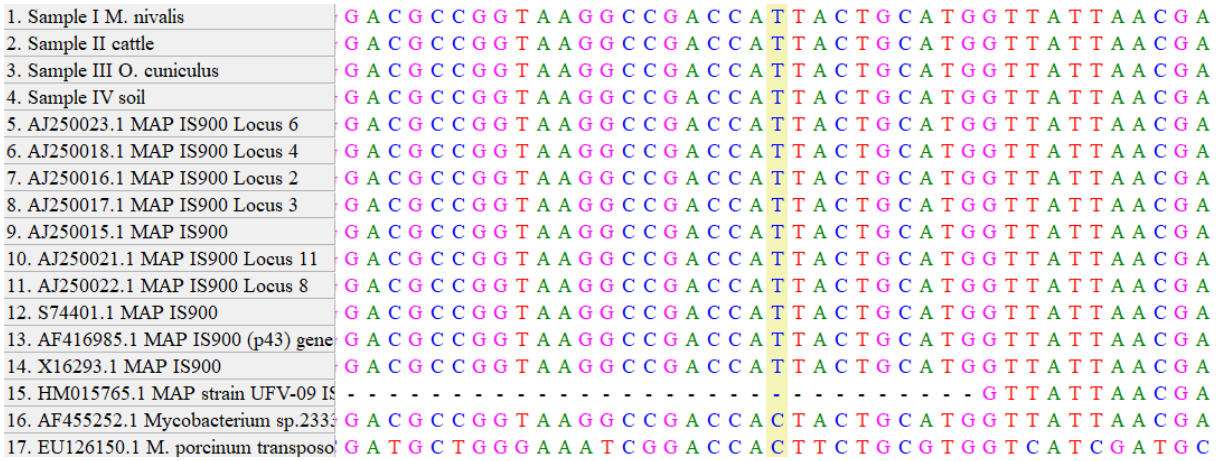

Figure 3.10: Nucleotide sequence alignment of two 224 bp amplicons from *M. nivalis* and cattle with eleven known IS*900* sequences (accession numbers AJ250023.1, AJ250018.1, AJ250016.1, AJ250017.1, AJ250015.1, AJ250021.1, AJ250022.1, S74401.1, AF416985.1, X16293.1 and HM015765.1) **and two IS900-like sequences** (accession number AF455252.1 and EU126150.1). Sequences retrieved from NCBI nucleotide database (accessed on August 2021). The SNP (T > C) that distinguishes IS*900*-like from MAP IS*900* is highlighted. Alignment was performed with MEGA-X (version 10.2.6).

3.3. Factors influencing MAP-infection in wildlife

The data did not present spatial autocorrelation (Moran's I = -0.035; *p*-value = 0.411), indicating that there is little influence of the spatial dimension in the data. However, since MAP prevalence may vary between species, this variable (i.e. species) was used as a random effect for all GLMMs to account for the underlying data structure.

In dataset A, data from all wild mammal species with at least 10 samples analyzed were considered. This included *O. cuniculus*, *V. vulpes*, *M. meles*, *E. europaeus*, *S. scrofa*, *L. granatensis*, *M. foina* and *M. nivalis*. Multicollinearity was confirmed for the land cover variables rice field, eucalyptus, irrigation plot and *montado* which were then discarded from the corresponding hypothesis due to high VIF values (VIF > 5). The best model (ika + diversity) reached an AICc of 197.1 (Table 3.1) and an AUC of 0.6524.

Table 3.1: Best models for each hypothesis within dataset A (All species with *n* > 10). df – degrees of freedom; AICc – Akaike's information criterion; ΔAICc – difference to the lowest AICc value; AICc weight – Akaike weights. Variable abbreviations are shown in Table 2.2.

Models	df	AICc	ΔAICc	AICc weight
H1 – Climate				
(Intercept)	2	203.2	0.00	0.394
hum	3	203.9	0.69	0.278
temp	3	204.9	1.71	0.167
rain	3	205.0	1.79	0.161
H2 – Land Use				
olive + shrub	4	202.0	0.00	0.15
olive	3	202.2	0.22	0.134
olive + m_pine	4	202.4	0.43	0.121
olive + shrub + m_pine	5	202.4	0.48	0.118
shrub	3	202.7	0.70	0.106
olive + shrub + flood	5	203.7	1.79	0.061
shrub + m_pine	4	203.8	1.81	0.060
olive + flood	4	203.9	1.91	0.058
olive + m_pine + flood	5	203.9	1.92	0.057
olive + shrub + u_pine	5	203.9	1.99	0.055
H3 – Host abundance				
ika + diversity	4	197.1	0.00	1.000
H4 – Aggregation points				
(Intercept)	2	203.2	0.00	0.485
feeders	3	204.1	0.87	0.314
water	3	205.0	1.75	0.202
H5 – Environmental MAP contamination				
(Intercept)	2	203.2	0.00	0.505
map cattle	3	204.6	1.41	0.250
map soil	3	204.7	1.45	0.245

The best model obtained for dataset A included two significant variables from the host abundance hypothesis: wildlife abundance (*p*-value = 0.033645; 95%CI: -3.564/-0.277) and wildlife diversity (*p*-value = 0.030759; 95% CI: 0.153/2.690) (Table 3.2).

Table 3.2: Output of the best model for dataset A (All species with *n* > 10). SE – standard error, *z* – statistic, *p* – *p*-value, 95% CI – confidence interval. Variable abbreviations are shown in Table 2.2.

Variable	Estimate	SE	*z*	*p*	95% CI
H3 – Host abundance					
(Intercept)	-1.1504	0.4157	-3.618	0.000297	-2.378/ -0.721
ika	-1.6735	0.7878	-2.214	0.033645	-3.564/ -0.277
diversity	1.3873	0.6422	2.160	0.030759	0.153/ 2.690

In dataset B, data from all carnivore species were considered. Multicollinearity was confirmed for the land cover variables olive grove, *montado*, eucalyptus and shrubland, which were then discarded from the corresponding hypothesis due to high VIF values (VIF > 5). The best model (mixed + rip) reached an AICc of 102.2 (Table 3.3).

Table 3.3: Best models for each hypothesis within dataset B (Carnivores). df – degrees of freedom; AICc – Akaike's information criterion; ΔAICc – difference to the lowest AICc value; AICc weight – Akaike weights. Variable abbreviations are shown in Table 2.2.

Models	df	AICc	ΔAICc	AICc weight
H1 – Climate				
(Intercept)	2	108.5	0.00	0.360
hum	3	108.9	0.39	0.296
temp	3	109.6	1.13	0.205
rain	3	110.4	1.89	0.140
H2 – Land Use				
mixed + rip	4	102.2	0.00	0.402
mixed + rip + open	5	103.5	1.26	0.215
mixed + rip + m_pine	5	103.7	1.47	0.193
mixed + rip + pivot	5	103.7	1.50	0.190
H3 – Host abundance				
(Intercept)	2	108.5	0.00	0.373
diversity	3	109.3	0.77	0.254
ika	3	109.6	1.10	0.215
cattle	3	110.2	1.72	0.158
H4 – Aggregation points				
(Intercept)	2	108.5	0.00	0.425
water	3	109.7	1.23	0.230
hum_areas	3	110.3	1.75	0.177
feeders	3	110.3	1.85	0.169
H5 – Environmental MAP contamination				
(Intercept)	2	108.5	0.00	0.483
map_soil	3	109.5	0.96	0.298
map_cattle	3	110.1	1.59	0.219

The best model obtained for dataset B included one significant variable, mixed forest cover (p-value = 0.00568; 95% CI: 2.555/14.985), and one variable with a p-value < 0.1, riparian habitat cover (p-value = 0.07557; 95% CI: -6.397/0.313) (Table 3.4). Other non-significant variables (open habitat, maritime pine and irrigation plot coverage) were included in other models with a ΔAICc < 2 and were then included in the average model (Table 3.4) that reached an AUC of 0.689.

Table 3.4: Output of the best model for dataset B (Carnivores). SE – standard error, z – statistic, p – p-value, 95% CI – confidence interval. (Variable abbreviations are shown in Table 2.2).

Variable	Estimate	SE	*z*	*p*	95% CI
H2 – Land Use					
(Intercept)	-1.3588	0.3185	4.216	0.00002	-1.99/ -0.727
mixed	8.7697	3.1299	2.766	0.00568	2.555/ 14.985
rip	-3.0418	1.6896	1.777	0.07557	-6.397/ 0.313
open	2.4093	2.3968	0.992	0.32113	-2.350/ 7.169
m_pine	-0.7026	0.8333	0.832	0.40535	-2.357/ 0.952
pivot	1.8636	2.1070	0.873	0.38268	-2.320/ 6.048

In dataset C, data from all lagomorph species were considered. Multicollinearity was confirmed for the cover variables rice field, eucalyptus, *montado* and irrigation plot, which were then discarded from the corresponding hypotheses due to high VIF values (VIF > 5). The best model from the land use hypothesis (mixed + olive) reached an AICc of 74.6 (Table 3.5). The only model from the host abundance hypothesis (ika) reached an AICc of 71.1 (Table 3.5) and an AUC of 0.7368. The best model from the environmental MAP contamination hypothesis (map_cattle) reached an AICc of 77.0 (Table 3.5).

Table 3.5: Best models for each hypothesis within dataset C (Lagomorphs). df – degrees of freedom; AICc – Akaike's information criterion; ΔAICc – difference to the lowest AICc value; AICc weight – Akaike weights. Variable abbreviations are shown in Table 2.2.

Models	df	AICc	ΔAICc	AICc weight
H1 – Climate				
hum + temp	4	78.2	0.00	0.487
hum + temp + rain	5	79.2	0.98	0.299
temp	3	79.9	1.64	0.214
H2 – Land Use				
mixed + olive	4	74.6	0.00	0.312
mixed + olive + shrub	5	75.9	1.38	0.157
mixed + olive + rip	5	76.0	1.47	0.149
mixed + olive + m_pine	5	76.1	1.57	0.142
mixed + olive + u_pine	5	76.4	1.89	0.121
mixed + olive + open	5	76.5	1.94	0.118
H3 – Host abundance				
ika	3	71.1	0.00	1.000
H4 – Aggregation points				
(Intercept)	2	80.9	0.00	0.547
hum_areas	3	82.6	1.70	0.234
feeders	3	82.8	1.83	0.219
H5 – Environmental MAP contamination				
map_cattle	3	77.0	0.00	0.649
map_cattle + map_soil	4	78.2	1.23	0.351

There were multiple hypotheses with significant variables in dataset C. The best model from the land use hypothesis included two variables with a *p*-value < 0.1: mixed forest (*p*-value = 0.0906; 95%CI: -8.595/0.630) and olive grove cover (*p*-value = 0.0911; 95% CI: -4.539/0.337) (Table 3.6). Besides these variables, the average model included non-significant variables (shrubland, riparian habitat, maritime pine, umbrella pine and open habitat cover) (Table 3.6), reaching an AUC of 0.7884. The only model from the host abundance hypothesis included one significant variable, wildlife abundance (*p*-value = 0.00432; 95%C I: -5.772/-1.255) (Table 3.6). The best model from the environmental MAP contamination hypothesis included one significant variable, MAP in cattle (*p*-value = 0.0312; 95% CI: -6.928/-0.327) (Table 3.6). MAP in soil was also included in the average model (Table 3.6) that reached an AUC of 0.7237.

Table 3.6: Output of the best models for dataset C (Lagomorphs). SE – standard error, *z* – statistic, *p* – *p*-value, 95% CI – confidence interval. Variable abbreviations are shown in Table 2.2.

Variable	Estimate	SE	*z*	*p*	95% CI
H2 – Land Use					
(Intercept)	-0.6032	0.3554	1.669	0.0951	-1.311/ 0.105
mixed	-3.9828	2.3114	1.693	0.0904	-8.593/ 0.627
olive	-2.1016	1.2221	1.690	0.0911	-4.539/ 0.337
shrub	1.0947	1.1356	0.948	0.3436	-1.171/ 3.360
rip	-2.4762	3.4512	0.705	0.4809	-9.361/ 4.401
m_pine	-1.1021	1.3679	0.792	0.4286	-3.831/ 1.627
u_pine	-0.6967	1.1126	0.615	0.5384	-2.916/ 1.523
open	-1.0206	1.7935	0.559	0.5761	-4.599/ 2.557
H3 – Host abundance					
(Intercept)	0.0878	0.4717	0.186	0.8524	-0.850/ 1.030
ika	-3.1712	1.1113	-2.854	0.0043	-5.772/ -1.255
H5 – Environmental MAP contamination					
(Intercept)	-0.8496	0.4027	2.077	0.0378	-1.651/ -0.048
map_cattle	-3.6276	1.6546	2.154	0.0312	-6.928/ -0.327
map_soil	0.5974	0.5971	0.983	0.3256	-0.594/ 1.788

Finally, in dataset D, only data from *O. cuniculus* was considered since it represents 30% of all wildlife data. Multicollinearity was confirmed for variables temperature, and irrigation plot, riparian habitat, open habitat, rice field and eucalyptus cover which were then discarded from the corresponding hypotheses due to high VIF values (VIF > 5). The best model from the land use hypothesis (mixed + shrub) reached an AICc of 57.8 (Table 3.7). The best model from the host abundance hypothesis (ika) reached an AICc of 55.6 (Table 3.7). The best model from the environmental MAP contamination hypothesis (map_cattle) reached an AICc of 60.4 (Table 3.7).

Table 3.7: Best models for each hypothesis within dataset D (*O. cuniculus*). df – degrees of freedom; AICc – Akaike's information criterion; ΔAICc – difference to the lowest AICc value; AICc weight – Akaike weights. Variable abbreviations are shown in Table 2.2.

Models	df	AICc	ΔAICc	AICc weight
H1 – Climate				
(Intercept)	1	63.0	0.00	0.655
hum	2	64.3	1.28	0.345
H2 – Land Use				
mixed + shrub	3	57.8	0.00	0.238
mixed + shrub + u_pine	4	59.1	1.23	0.129
mixed + shrub + montado	4	59.2	1.34	0.122
shrub	2	59.2	1.35	0.121
shrub + montado	3	59.3	1.50	0.113
shrub + u_pine	3	59.7	1.87	0.094
mixed + shrub + flood	4	59.7	1.91	0.092
shrub + u_pine + montado	4	59.7	1.91	0.092
H3 – Host abundance				
ika	2	55.6	0.00	0.547
ika + diversity	3	56.0	0.38	0.453
H4 – Aggregation points				
(Intercept)	1	63.0	0.00	0.434
hum_areas	2	64.5	1.48	0.207
water	2	64.6	1.62	0.193
feeders	2	64.9	1.92	0.166
H5 – Environmental MAP contamination				
map_cattle	2	60.4	0.00	0.693
map_cattle + map_soil	3	62.0	1.63	0.307

There were multiple hypotheses with significant variables in dataset D. The best model from the land use hypothesis included one significant variable, shrubland cover (p-value = 0.04139; 95% CI: 0.126/ 6.306) and a non-significant variable, mixed forest cover (Table 3.8). Other land use variables (umbrella pine, *montado* and floodplain cover) were included in the average model (Table 3.8) that reached an AUC of 0.7508. The best model from the host abundance hypothesis included one significant variable, wildlife abundance (p-value = 0.0158; 95%CI: -5.367/-0.556) (Table 3.8). Wildlife diversity was included in the average model that reached an AUC of 0.7342 (Table 3.8). The best model from the environmental MAP contamination model included one variable with a p-value < 0.1, MAP in cattle (p-value = 0.0559; 95%CI: -7.208/0.0895) (Table 3.8). MAP in soil was also included in the average model that reached an AUC of 0.7158 (Table 3.8).

Table 3.8: Output of the best models for dataset D (*O. cuniculus*). SE – standard error, *z* – statistic, *p* – *p*-value, 95% CI – confidence interval. Variable abbreviations are shown in Table 2.2.

Variable	Estimate	SE	*z*	*p*	95% CI
H2 – Land Use					
(Intercept)	-1.7325	0.6089	2.808	0.00498	-2.942/ -0.523
mixed	-2.6570	2.0442	1.273	0.20304	-6.748/ 1.434
shrub	3.2160	1.5455	2.040	0.04139	0.126/ 6.306
u_pine	2.7608	2.2223	1.217	0.22352	-1.685/ 7.206
montado	1.1946	0.9614	1.219	0.22302	-0.727/ 3.116
flood	4.0362	6.2919	0.628	0.52993	-8.558/ 16.631
H3 – Host abundance					
(Intercept)	-0.4330	0.9479	0.450	0.6525	-2.318/ 1.452
ika	-2.9614	1.2026	2.413	0.0158	-5.367/ -0.556
diversity	2.0216	1.5072	1.314	0.1889	-0.994/ 5.038
H5 – Environmental MAP contamination					
(Intercept)	-0.9562	0.4253	2.205	0.0275	-1.806/ -0.106
map_cattle	-3.5594	1.8241	1.912	0.0559	-7.208/ 0.0895
map_soil	0.5221	0.6840	0.748	0.4546	-0.846/ 1.891

All estimated average models aimed at representing the most parsimonious and explanatory hypothesis to describe the presence/absence of MAP in the corresponding dataset.

In short, mixed forest and shrubland cover showed a significant positive influence on MAP infection in carnivores and *O. cuniculus*, respectively; wildlife abundance showed a significant negative influence on MAP infection in all species with $n > 10$, lagomorphs and *O. cuniculus*; and MAP in cattle showed a significant negative influence on MAP infection in lagomorphs (Figure 3.11). Other variables, such as riparian habitat cover for carnivores, mixed forest and olive grove cover for lagomorphs, and MAP in cattle for *O. cuniculus* showed no significant influence although they can be important variables, since they were included in the best models and have a *p*-value lower than 0.1 (Figure 3.11).

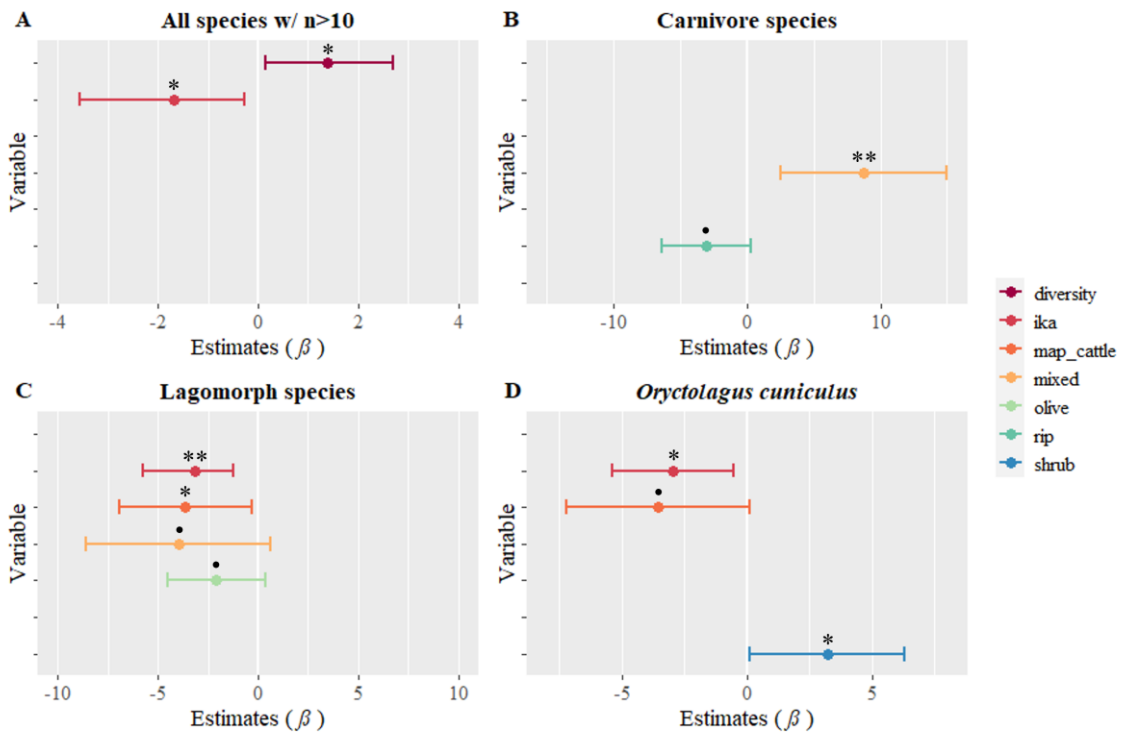

Figure 3.11: Summary of the outputs of the significant variables of the best models for each dataset. Estimates of the fixed effects with the associated 95% confidence intervals. • – *p*-value < 0.1; ∗ – *p*-value < 0.05; ∗∗ – *p*-value < 0.01. Variable abbreviations are shown in Table 2.2.

3.4. Prediction of high MAP infection risk areas

To predict areas of high MAP infection risk, a new model was fitted with the explanatory variables with a *p*-value lower than 0.1 from all previous models (Figure 3.11). Here, we included wildlife abundance, wildlife diversity, MAP prevalence in cattle, and mixed forest, riparian habitat, shrubland and olive grove cover, since they showed no multicollinearity. Wildlife abundance (*p*-value = 0.026; 95% CI: -4.386/-0.280) and mixed forest coverage (*p*-value = 0.041; 95% CI: 0.080/3.915) were the only significant variables, with negative and positive influence, respectively (Table 3.9). Wildlife diversity showed a *p*-value lower than 0.1 but registered no significant influence (*p*-value = 0.077; 95% CI: -0.125/2.417) (Table 3.9). Although the remaining variables showed a *p*-value higher than 0.1, they were used to predict areas of high MAP infection risk since they were included in the average model (Table 3.9). The model reached an AUC of 0.661, which indicates a low prediction accuracy.

Table 3.9: **Output of the average model built for mapping the risk of MAP infection to wildlife.** SE – standard error, *z* – statistic, *p* – *p*-value, 95% CI – confidence interval. Variable abbreviations are shown in Table 2.2.

Variable	Estimate	SE	*z*	*p*	95% CI
(Intercept)	-1.131	0.4111	2.738	0.00617	-1.940/ -0.321
diversity	1.1465	0.6447	1.768	0.07707	-0.125/ 2.417
ika	-2.3327	1.0425	2.227	0.02594	-4.386/ -0.280
mixed	1.9978	0.9735	2.042	0.04115	0.080/ 3.915
rip	-1.6393	1.2669	1.286	0.19828	-4.137/ 0.858
shrub	1.5275	1.108	1.37	0.1706	-0.657/ 3.712
map_cattle	-0.8048	0.7129	1.122	0.26183	-2.210/ 0.601
olive	1.5412	1.5984	0.959	0.33777	-1.610/ 4.692

The risk of wildlife MAP infection in our study area was mapped and classified according to the probability of infection, which ranged between 20% and 80%, represented in Figure 3.12A as the lightest and darkest shades, respectively. There are two main predicted hotspots for infection risk, X and Y (Figure 3.12A). Although these hotspots do not fully overlap with the observed areas with the highest MAP prevalence in wildlife, they seem to be located near these areas (Figure 3.12B).

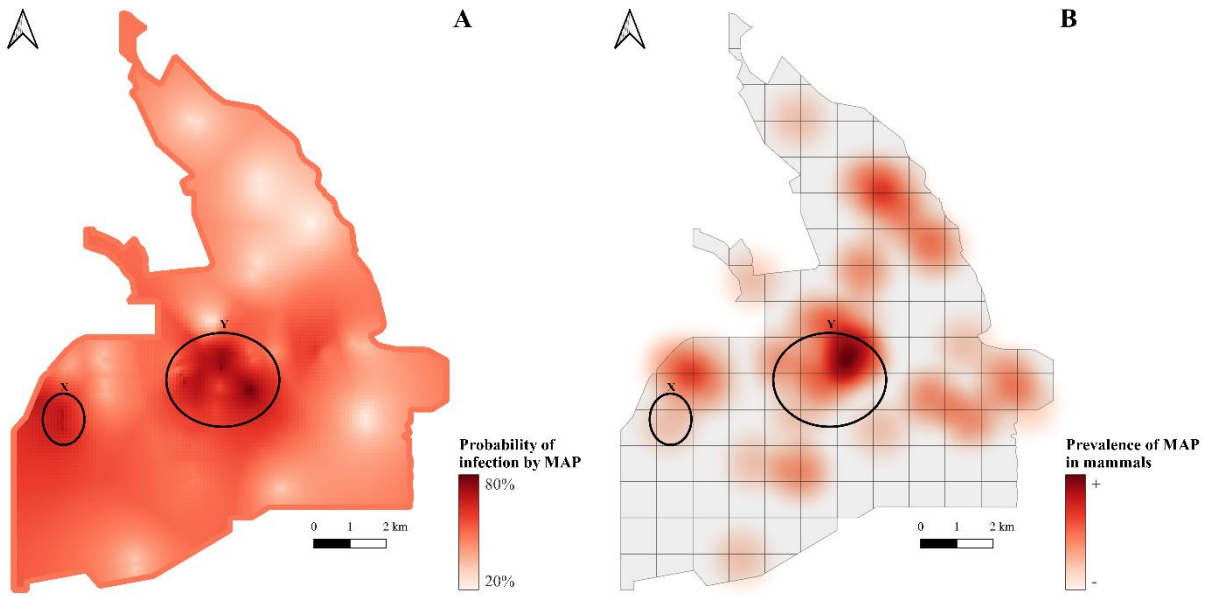

Figure 3.12: Hotspots of MAP infection risk in Charneca do Infantado. (A) Prediction of areas with high MAP infection risk estimated from the logistic regression model with the variables selected; (B) heatmap of the number of wildlife positive samples for MAP infection. Infection hotspots are circled in black and labeled.

4. DISCUSSION

Knowledge about the transmission paths of MAP remains scarce. Thus, it is important to collect information on the factors that may increase the risk of infection and transmission between livestock and wildlife. Transmission between livestock and wildlife might result from the ingestion of contaminated fecal material[23]; and so, environmental contamination arising from fecal shedding of bacteria by infected animals may be an important reservoir of MAP, which can then play a major role on infection through ingestion of contaminated vegetation and water.

In this work, we collected and analyzed 206 samples from mammals belonging to 11 species. Most species were well distributed across the study area, except for *O. cuniculus* (Figure 3.2). Still, the overall sample distribution per species was not homogenous, varying between four *G. genetta* and *L. lutra* samples, and 62 *O. cuniculus* samples. The rabbit population in CL is fragmented and has been declining over the years, especially due to epizootics caused by viral hemorrhagic disease, even though it was considered locally abundant in 2013[51]. Rabbits are social animals and live in colonies[51], and as such, multiple latrines may be found in the same area, resulting in higher numbers of samples in specific transects. Latrines are not exclusive of each individual; however, for the purposes of our analyses, latrines were considered as a single sample. Thus, MAP prevalence may be under- or overestimated, depending on whether the analyzed pellets were from a healthy or infected individual, respectively. Similarly, badgers (*M. meles*) are also social animals, and latrines are commonly found next to each other, thus, the identification of feces down to the individual level is impaired[59]. Given these constraints, we collected fecal material from each latrine found together and combined it into a single sample.

Wild mammals are scarce and elusive animals, most being nocturnal, and so their sampling carries some difficulties[72]. Non-invasive sampling, like collection of feces found in the environment, is a cost-effective way to sample mammals and avoid unnecessary stress to animals. However, this means that the samples collected are dependent on the field observer, resulting in a heterogeneous sampling. Furthermore, most samples found in the environment suffer from autolysis and degradation because of their prolonged exposure to abiotic and biotic stressors. In our study, we used morphological methods to identify scats down to the species level, which could cause potential misidentification and contribute to result bias. Inaccurate identification of scats in carnivores can vary from 14% to 88%[73], hence molecular analysis of scats for the identification of species origin should be done whenever possible.

MAP was detected in wildlife (in 10 out of the 11 sampled species), cattle (in 100% of the herds sampled) and soil. Since the number of samples was limited for most species, we are not able to estimate prevalence within each species; however, we can confirm that exposure to MAP exists. The overall MAP prevalence in wildlife (22%) seems to be consistent with data from previous studies in Portugal[11,45–47]. Differences were mainly observed between our results for *O. cuniculus* (19.35%) and those from another study, which analyzed prevalence by ELISA (Enzyme-Linked Immunosorbent Assay) (7.9%[46]). These discrepancies may arise from the sampling strategy (pooled samples in the case of social animals that defecate in latrines versus individual samples in ELISA); the type of biological specimen (feces versus blood serum) and the information it provides (feces enable isolation of the pathogen or specific DNA detection of the latter, while serum gives information on antibodies, indicating exposure to the pathogen); or the performance of each methodological approach (molecular versus serological). Even though *O. cuniculus* is not a ruminant species, the high MAP prevalence observed was not unexpected. This species is considered a wildlife reservoir, since, when compared with other non-ruminant species, it shows a high burden of infection in tissues and excretes high quantities of bacteria through feces[74]. High prevalence of MAP in prey species, like rodents and rabbits, increases the risk of infection of carnivore species that prey on these infected animals. Thus, the high prevalence observed in *V. vulpes* and the presence of MAP in most surveyed carnivore species was not unexpected. This study represents the first

evidence of MAP infection in *Erinaceus europaeus* in Portugal, although previous studies in other regions of the world had confirmed infection in this species[75].

The overall high detection rate of MAP across the surveyed mammals might be explained by the proximity between cattle and wildlife in our study area. In Portugal, previous studies have found low prevalence levels of MAP in cattle analyzed by ELISA (2.3-7%[40,43]). However, we observed a much higher prevalence in our study (54%). In general, ELISA methods seem to be much less sensitive than molecular analysis[76], possibly explaining these differences, and making it difficult to compare results. Also, the studies available so far were focused on different regions, while this was the first time that CL was monitored for this pathogen. Some factors associated with farm management and individual animal characteristics may increase prevalence of MAP in cattle[77]. Çetinkaya and collaborators (1997)[77], for example, found that farms with a predominance of a particular breed of cattle may show higher MAP infection. Unfortunately, there is no information in the literature regarding this issue on the breeds screened in our study. In CL, the constant movement of herds during our sampling made it difficult to assess the true influence of cattle in the presence of MAP in wildlife. Furthermore, these movements might increase MAP transmission inside the herd and between herds, and promote MAP dispersion across the study area.

This study is the first to confirm soil contamination by MAP in Portugal (44%). When possible, soil samples were collected near water sources, e.g. puddles and ponds, which suggests that water sources are also contaminated. However, MAP was not detected in any of the fecal samples of *L. lutra*, a predominantly aquatic species. Salgado and collaborators (2011)[78] showed experimentally that MAP remains in the upper soil layers and grass, confirming the danger for transmission to animals, especially herbivores that consume this contaminated vegetation. Moreover, with heavy rain, runoff to water sources may happen, furthering the transmission cycle. This may represent an important source of transmission, even to humans. Sousa and collaborators (2021)[79], for example, have detected a high burden of MAP in municipal and domestic water in the Porto area, the second largest city of Portugal. Since we detected such a high prevalence of MAP in soil samples, we can confirm that environmental contamination exists, and thus, intra- and interspecific transmission may exist through this route in our study area.

We studied factors associated with climate, land use, host abundance, aggregation points and MAP environmental contamination in order to understand how they modulate the risk of MAP infection in wildlife and thus explain the high prevalence observed in our study area. Contrarily to what we expected, MAP in soil did not have any influence on MAP detection in wildlife. This result could indicate a low sampling effort, since only one sample from each transect was collected. Sampling of other environmental matrices, such as herbaceous vegetation and watercourses, could have different results and contribute to a better understanding of the influence of environmental contamination.

Furthermore, we observed an unexpected result when focusing the modeling analyses on datasets C and D, since MAP in cattle showed a negative influence on MAP presence in lagomorphs, while we expected the opposite effect. There is no evidence that rabbits avoid pastures grazed by cattle; in fact, it is possible that they take advantage of these pastures, since they prefer shorter vegetation[80]. However, rabbits might avoid cattle feces and, thus, not ingest infected vegetation. Studies have shown that cattle do not avoid vegetation contaminated with rabbit feces[36], but the opposite remains to be studied.

The variable that showed the largest influence on datasets A, C and D, was wildlife abundance, with a negative trend. Once again, this result was unexpected, as a higher abundance should increase contact between animals, and consequently increase intra- and interspecific transmission of MAP. Since this trend seems to be more correlated with lagomorphs (datasets C and D), this result could reflect an avoidance of areas with high abundance of predator species, which represent most of our data.

The variables that showed a positive influence on MAP detection were wildlife diversity in dataset A, mixed forest coverage in dataset B, and shrubland coverage in dataset D. The mixed forest habitat

promotes high diversity and abundance of animals, especially carnivores, because of its heterogeneity[81]. Furthermore, the shrubland is known to be a preferred habitat of rabbits, since it provides shelter from predators[60]. In CL, rabbits are observed predominantly in areas with abundance of *Cistus ladanifer*, a shrub native to the Mediterranean region. Thus, these results show, in contrast with the previous findings, that abundance and diversity of animals might contribute to their infection. This ambiguity might be related with the fact that the latter results concern a species or a group of species that are similar (taxonomical family), while a different trend is observed when considering the different species altogether. This shows the importance of studying variables particular to each species or group, while considering the different behaviors and preferences, and, in particular, the different paths of infection. For this purpose, large datasets of samples are needed in order to evenly represent all species.

On the other hand, given the non-invasive nature of this study in wild animals, the viability of MAP (indispensable for infection) in areas where its presence has been detected through environmental DNA is unknown, and so we cannot accurately estimate the infection status of animal populations in these contaminated areas from the data currently available.

Finally, we sought to predict the spatial risk of MAP infection in wildlife across the study area, using the most important explanatory variables within each dataset to extrapolate beyond the sampled areas. The results showed two main hotspots reaching 80% of risk of infection and, overall, our model seems to predict high infection risk across the study area (Figure 3.12). However, the model used had insufficient support (AUC = 0.661). Even though the variables chosen to build this model showed some influence for each dataset (Figure 3.11), most of the obtained models also showed low accuracy (AUC < 0.7).

During statistical analysis, other modeling approaches were tested; however, their results were deemed irrelevant or redundant, and therefore were not included in the final work.

We can conclude that the variables considered were not enough to accurately extrapolate the risk of infection to non-observed areas. Further studies are needed to accurately predict infection risk in our study area. Increasing the number of samples and sampled species, as well as considering new variables, would be beneficial. Furthermore, increasing the number of environmental samples, e.g. soil and vegetation, as well as analyzing watercourses, could increase the level of available information.

This study has advanced knowledge on the distribution of MAP infection by screening the mammal community of Companhia das Lezírias, providing opportunities for intervention, including adaptive management measures to mitigate infection risk in cattle, and also increasing the level of information available at a national level. Furthermore, it confirmed the presence of MAP in soil, suggesting it could be an indirect source of infection for domestic and wild animals.

5. CONCLUSION AND FUTURE PERSPECTIVES

This study allowed us to estimate the occurrence rates of MAP in different populations, at a local scale in a Mediterranean agro-forestry farmstead. Since different studies use different methods for MAP detection, comparison of results between different areas of Portugal is impaired. A global systematic method should be applied in order to have a holistic understanding of the scale of MAP infection in wildlife and livestock in Portugal.

Unfortunately, we were not able to obtain enough data to understand the factors that may increase infection risk in wildlife and accurately predict areas with higher risk. Variables that showed potential influence are related to land use, abundance and diversity of wildlife and cattle. Spatial inferences can be difficult to implement in local studies that include small areas. Although our study area has a large diversity of land uses, most of its area is covered by *montado*. Therefore, landscape variability in our study was insufficient between each sampled transect to be able to determine which factors would increase risk of infection. Future studies should include different areas in order to compare results from different climates, different habitats, areas with and without cattle, areas with other livestock, and areas with intensive and extensive livestock production.

Moreover, many small mammal species were missing from our sampling. Studies have shown that rodents might be infected through ingestion of contaminated feed[33]. However, in our study area, cattle are managed in extensive systems, with no addition of feed. Here, rodents may become infected through consumption of acorns and other seeds found in soil contaminated with cattle feces. These small mammals represent a big part of the diet of carnivore species; thus, their study is important to understand their role on MAP transmission. We were able to sample an insectivorous species, *Erinaceus europaeus*, and find MAP DNA, which supports the idea that animals may become infected by consuming insects. Recently, a new rodent species has been recorded in our study area, the red squirrel (*Sciurus vulgaris*). According to the available information, MAP occurrence has not yet been reported or screened in this species, either in Portugal or in the rest of the world. Although squirrels are mainly arboreal, they use the ground for feeding purposes[82]. Thus, similarly to other rodent species, these animals might also be at risk of infection. Moreover, the presence of a cervid species (Fallow deer, *Dama dama*) was also recorded recently in CL. This species is the only wild ruminant present in our study area and exists only in small numbers, and should be taken into consideration in future studies.

In addition to a molecular approach based on environmental DNA analysis, a cultural approach is also suggested in future studies, in order to determine the viability of MAP in the analyzed biological matrices, despite the methodological constraints for the culture and isolation of these rather fastidious bacteria. Only then, if maintaining a non-invasive sampling approach, will it be possible to establish a link between the detection of MAP DNA, MAP viability, and probability of animal infection.

Control and eradication of paratuberculosis remains a challenge, since there are no cost-effective treatments or vaccines able to prevent infection[83]. Some vaccines have shown great results, with significant decrease of bacteria shedding in cattle[83,84]. Vaccination methods are increasingly less expensive and more sustainable[84], so they should be used when possible. Still, testing and culling continues to be the method that is generally used to control infection by MAP in livestock, but the inherent losses are large and detection of infection is difficult[84]. On the other hand, efforts to decrease infection in livestock are hindered by the presence of infection in wildlife. Fox and collaborators (2018)[85] have reported that efforts to decrease infection in cattle with a test-and-cull strategy do not reduce infection in rabbits, which seem to be an important MAP reservoir contributing to the persistence of the infection[85,86]. If MAP is already established in wildlife populations, control measures for livestock should be accompanied by actions to control the infection in wild animals. However, an integrated control strategy should be carefully planned, since rabbits are endangered in Portugal, and so, culling programs are unadvised.

6. REFERENCES

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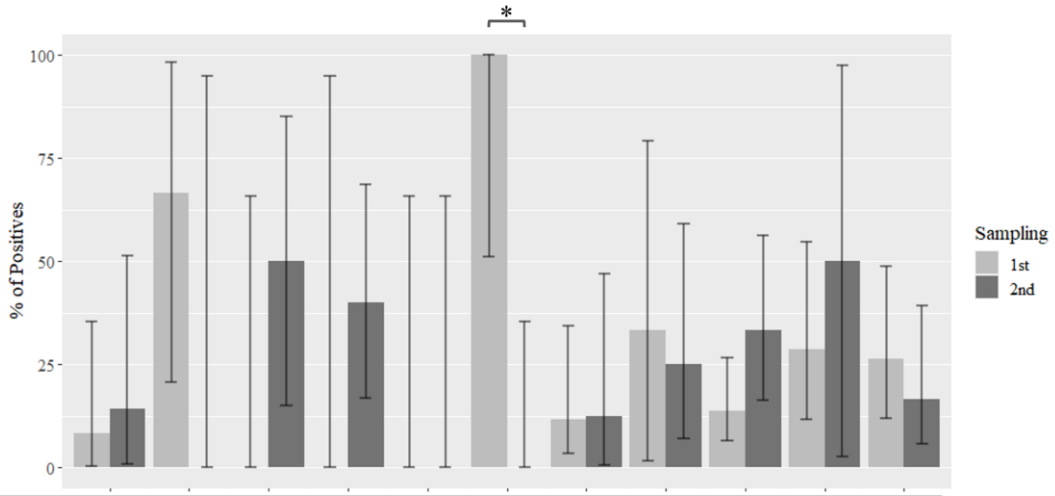
APPENDIXES

Supplementary table 1 – Species that occur in Charneca do Infantado, Companhia das Lezírias and their conservation status (DD – Data Deficient, LC – Least Concern, NT – Near Threatened, VU – Vulnerable, EN – Endangered). IUCN - International Union for Conservation of Nature[87], LVPT - *Livro Vermelho dos Vertebrados de Portugal*[52].

Order	Species	Common name	Conservation status	
			IUCN	LVPT
Eulipotyphla	*Erinaceus europaeus*	European hedgehog	LC	LC
	Crocidura russula	Greater white-toothed shrew	LC	LC
	Suncus etruscus	White-toothed pygmy shrew	LC	LC
	Talpa occidentalis	Iberian mole	LC	LC
Lagomorpha	*Oryctolagus cuniculus*	European rabbit	EN	NT
	Lepus granatensis	Iberian hare	LC	LC
Rodentia	*Microtus cabrera*	Cabrera's vole	NT	VU
	Microtus lusitanicus	Lusitanian pine vole	LC	LC
	Apodemus sylvaticus	Wood mouse	LC	LC
	Rattus norvegicus	Brown rat	LC	NA
	Mus spretus	Algerian mouse	LC	LC
	Sciurus vulgaris	Eurasian red squirrel	LC	LC
Carnivora	*Vulpes vulpes*	Red fox	LC	LC
	Mustela nivalis	Least weasel	LC	LC
	Mustela putorius	Western polecat	LC	DD
	Martes foina	Stone marten	LC	LC
	Meles meles	Eurasian badger	LC	LC
	Lutra lutra	Eurasian otter	LD	LC
	Genetta genetta	Common genet	LC	LC
	Herpestes ichneumon	Egyptian mongoose	LC	LC
	Felis silvestris	Wild cat	LC	VU
Cetartiodactyla	*Sus scrofa*	Wild boar	LC	LC
	Dama dama	Fallow deer	LC	NA



Species		EE	GG	HI	LG	LL	MF	MM	MN	OC	SS	VV
Nº samples	1st sampling	12	3	2	1	2	4	17	3	44	14	19
	2nd sampling	7	1	4	10	2	7	8	8	18	2	18

Supplementary Figure 1: Percentage of positive samples for each wildlife species per sampling period with the associated 95% confidence intervals. The asterisk represents significant differences ($*$ – p-value < 0.05).