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Iberian wild leporidae as hosts of zoonotic enteroparasites in Mediterranean ecosystems of Southern Spain

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Abstract

Wild lagomorphs including rabbits and hares can act as natural carriers or reservoirs of bacterial and parasitic zoonotic diseases. However, little is known on the epidemiology and potential public health significance of intestinal eukaryotes in wild leporids. We examined faecal samples from European wild rabbits (*Oryctolagus cuniculus*, *n* = 438) and Iberian hares (*Lepus granatensis*, *n* = 111) collected in the Autonomous Region of Andalusia in southern Spain during 2012–2021. We searched for the presence of DNA from the main intestinal protist and microsporidial pathogens of veterinary and public health concerns using molecular methods (PCR followed by Sanger and next-generation sequencing). *Giardia duodenalis* was the most prevalent species found (27.8%, 153/550; 95% CI: 24.1–31.8), followed by *Cryptosporidium* spp. (1.3%, 7/550; 95% CI: 0.5–2.6), *Blastocystis* sp. (1.1%, 6/550; 95% CI: 0.4–2.4) and *Encephalitozoon intestinalis* (0.2%, 1/550; 95% CI: 0.0–10.1). All samples tested negative for *Enterocytozoon bieneusi*. Sequence analyses revealed the presence of sub-assemblage BIV (*n* = 1) within *G. duodenalis*, and *Cryptosporidium cuniculus* (*n* = 6) and *Cryptosporidium andersoni* (*n* = 1) within *Cryptosporidium*. The presence of ruminant-adapted *C. andersoni* is indicative of a potential cross-species transmission event, although a spurious infection (mechanical carriage) cannot be ruled out. Samples assigned to *C. cuniculus* belonged to the *gp60* subtype families Va (*n* = 3) and Vb (*n* = 2). The six *Blastocystis*-positive samples were identified as ST2 (*n* = 3) and ST1+ST2 (*n* = 3). Our molecular results suggest that wild rabbits and hares were primarily infected by leporid-adapted species of eukaryotic pathogens. However, the occasional findings of zoonotic *G. duodenalis* sub-assemblage BIV, *Blastocystis* sp. ST1 and ST2, and *Encephalitozoon intestinalis* could be of public health relevance.

KEYWORDS
Blastocystis, *Cryptosporidium*, *Encephalitozoon*, epidemiology, genotyping, *Giardia*, Leporidae, microsporidia, transmission, zoonoses

Laura Rego and Sabrina Castro-Scholten contributed equally to this work.

1 | INTRODUCTION

The European wild rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*) are native mammals and keystone species of the Iberian Peninsula. They play an important role in the ecology and diversity of the Spanish Mediterranean ecosystems (Delibes-Mateos et al., 2007; Gortazar et al., 2007), being the staple prey for a large number of predators (Ferrer & Negro, 2004). Additionally, both species raise significant animal and public health concerns, since they are widely distributed in Iberia, are very adaptable to different ecological conditions, and share habitat and resources with other sympatric domestic and wild species, as well as humans. Leporids are also among the main small game species in Spain with about 6 million of wild rabbits and 391,000 hares hunted annually and that are generally consumed without sanitary inspection because they are intended for small-scale retail sale or personal consumption [Ministerio de Agricultura, Pesca y Alimentación (MAPA), 2022]. Additionally, wild leporids have been shown to be natural carriers or reservoirs of different zoonotic agents such as *Coxiella burnetii* (González-Barrio et al., 2015), *Francisella tularensis* (Mínguez-González et al., 2021), *Leishmania infantum* (Jiménez et al., 2014), *Rickettsia* spp. (Remesar et al., 2022), and *Toxoplasma gondii* (Almeria et al., 2021). Public health concerns highlight the need for epidemiological studies on zoonotic diseases affecting wild species that are a source of food for humans.

Little information is currently available on the epidemiology and potential health impact of zoonotic enteric eukaryotes in wild lagomorph populations. These include diarrhoea-causing enteric protozoans such as *Giardia duodenalis* and *Cryptosporidium* spp. (Baz-González et al., 2022; Marhoon et al., 2018; Robinson & Chalmers, 2010), the microsporidia *Enterocytozoon bieneusi* (Li et al., 2019) and *Encephalitozoon* spp. (Martínez-Padilla et al., 2020), and the stramenopile of uncertain pathogenic significance *Blastocystis* sp. (Li et al., 2020). All these intestinal eukaryotes share the faecal–oral transmission route, with infections being acquired either through direct contact with infected individuals or indirectly via ingestion of contaminated water or food. Remarkably, many of the species/genotypes/subtypes of the above-mentioned eukaryotes can be zoonotically transmitted (Dixon, 2021; Hublin et al., 2021; Li et al., 2019; Ryan et al., 2021). Table 1 summarizes the main molecular-based epidemiological features of intestinal protist and microsporidial pathogens described in wild lagomorph species in Europe.

Giardia duodenalis has a major public and animal health significance in terms of gastrointestinal disease, being able to infect a wide host range, including amphibians, birds, and mammals (Dixon, 2021; Feng & Xiao, 2011). *Giardia duodenalis* is currently recognized as a species complex comprising eight distinct genotypic assemblages (A to H) with marked differences in host specificity and range (Feng & Xiao, 2011). Zoonotic assemblage B is typically the most prevalent *G. duodenalis* genetic variant detected in farmed and pet rabbits (Jiang et al., 2018; Pantchev et al., 2014; Zhang et al., 2012), although assemblages A and E have also been sporadically documented in farmed and wild rabbits (Koehler et al., 2016; Qi et al., 2015).

Impacts

- This work is the largest European epidemiological study aiming at investigating the occurrence and genetic diversity of eukaryotic parasites in wild leporids.
- Wild rabbits and hares are primarily infected by host-adapted species/genotypes of the eukaryotic species under investigation, although they can also serve suitable reservoirs for zoonotic pathogens including *Cryptosporidium cuniculus* and *Blastocystis* ST1 and ST2.
- Evidence that overlapping transmission cycles of *Cryptosporidium* species (particularly *Cryptosporidium andersoni*) between domestic and wild habitats, and first description of the genetic diversity of *Blastocystis* sp. in wild leporids globally.

At least 44 *Cryptosporidium* species are currently recognized (Ryan et al., 2021), of which host-adapted *C. cuniculus* and, to a much lesser extent, *C. parvum* have been identified in farmed and wild lagomorphs (Ayinmode & Agbajelola, 2019; Koehler et al., 2016; Learmonth et al., 2004). Remarkably, *C. cuniculus* has been confirmed as the etiological agent of a waterborne outbreak of human diarrhoea in the UK (Puleston et al., 2014).

The stramenopile *Blastocystis* sp. is a ubiquitous intestinal protist that infects/colonizes a broad range of mammalian and avian species (Hublin et al., 2021). It is also considered the most widespread non-fungal microeukaryote present in human stool samples (Andersen & Stensvold, 2016). Thirty *Blastocystis* subtypes (ST1-ST17, ST21, ST23-ST34) have been formally described to date (Baek et al., 2022; Higuera et al., 2021; Maloney & Santin, 2021; Maloney et al., 2021; Stensvold & Clark, 2020), of which ST4 is the most frequently detected in farmed rabbits (Li et al., 2020; Wang et al., 2018). Sporadic infections by ST14 have also been detected in farmed and pet rabbits in the United Arab Emirates (AbuOdeh et al., 2019). No information is currently available on the molecular diversity of *Blastocystis* in wild leporids.

The phylum Microsporidia comprises more than 1500 obligate intracellular spore-forming parasites classified as fungi that can infect a wide range of vertebrate and invertebrate hosts through the faecal–oral route (Han et al., 2021; Snowden, 2014). Among the 17 zoonotic microsporidia species, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *E. hellem* and *E. cuniculi* are the most common species reported in humans (Qiu et al., 2019). The latter species is particularly adapted to infect lagomorph hosts. Foodborne, waterborne, and zoonotic transmission has previously been evidenced as a route of microsporidian infection (Cama et al., 2007; Decraene et al., 2012; Michlmayr et al., 2022). In this context, zoonotic microsporidia species have been detected in wild lagomorphs from European countries (Bártová et al., 2015; De Bosschere et al., 2007; Künzel & Joachim, 2010; Martínez-Padilla et al., 2020; Zanet et al., 2013). Genotyping *E. bieneusi* isolates from lagomorphs worldwide has

TABLE 1 Molecular-based data on the occurrence and genetic diversity of intestinal protist and microsporidial pathogens in wild lagomorph species in Europe

Pathogen	Host	Population status	Country	Infection rate % (no.pos./total)	Species identified (no.)	Genotype (no.)	Reference
Cryptosporidium spp.	Rabbit	Wild	Spain	1.0 (1/100)	*C. cuniculus* (1)	VbA26R3 (1)	Baz-González et al. (2022)
			The Netherlands	6.5 (2/31)	*Cryptosporidium* spp. (2)	ND	Robinson and Chalmers, (2010)
			UK	7.0 (2/28)	*C. parvum* (2)	ND	Sturdee et al. (1999)
		Farmed	Poland	NA	*C. cuniculus* (NA)	VbA24 (NA)	Kaupke et al. (2014)
			Czech Republic	NA	*C. cuniculus*(2)	ND	Ryan et al. (2003)
			Belgium	8.0 (12/149)	*Cryptosporidium* spp. (12)	ND	Peeters et al. (1986)
		Companion	UK	2.6 (1/38)	*Cryptosporidium* spp. (1)	ND	Smith et al. (2009)
Giardia duodenalis	Rabbit	Companion	Various[a]	7.6 (40/528)	–	Assemblage B (1)	Pantchev et al. (2014)
			Russia	1.8 (3/165)	–	ND	Kurnosova et al. (2019)
	Hare	Wild	Croatia	0.0 (0/73)	–	–	Beck et al. (2011)
Microsporidia	Rabbit	Wild	Spain	1.0 (4/383)	*E. bieneusi* (3)	ND	Martínez-Padilla et al. (2020)
					E. intestinalis (1)	–	
			Italy[b]	9.7 (14/144)	*E. cuniculi* (14)	ND	Zanet et al. (2013)
		Farmed	Spain	21.1 (4/19)	*E. bieneusi* (4)	D	Galván-Díaz et al. (2014)
		Companion	Slovakia	71.4 (10/14)	*E. cuniculi* (10)	Genotypes II (2) and III (4)	Valencakova et al. (2012)
			Spain	9.0 (2/22)	*E. cuniculi* (2)	ND	Lores et al. (2002)
			Switzerland[c]	100 (9/9)	*E. cuniculi* (9)	ND	Deplazes et al. (1996)
		Laboratory	Turkey	16.0 (19/119)	*E. cuniculi* (19)	Genotype I (19)	Ozkan et al. (2019)
	Hare	Wild	Spain	3.8 (3/79)	*E. intestinalis* (3)	–	Martínez-Padilla et al. (2020)
			Belgium[d]	100 (1/1)	*E. intestinalis* + *E. hellem* (1)	–	De Bosschere et al. (2007)

Abbreviations: NA, not available; ND, not determined.

[a]Including Germany and other European countries.

[b]Parasite detected in *Sylvilagus floridanus*.

[c]Previously identified as *Encephalitozoon cuniculi*.

[d]Case-report study.

revealed the presence of 19 genotypes (J, BEB8, Type IV, SC02, I, N, CHY1, D, CHN-RD1, Peru6, CHN-RR1 to CHN-RR3, SCR01, SCR02 and SCR04 to SCR07; Deng et al., 2020; Yang et al., 2016; Zhang et al., 2016, 2018).

Hence, based on this scarcity of data, this study was carried out to determine the prevalence, geographical distribution, genetic diversity, and zoonotic potential of intestinal microeukaryotic parasites in wild leporids (European wild rabbits and Iberian hares) from Mediterranean ecosystems of southern Spain.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

This study did not involve purposeful killing of animals. All samples were collected from legally hunted animals during the hunting seasons or by passive surveillance under Spanish and Andalusian legislation. No ethics approval was necessary.

2.2 | Study design and sampling

A cross-sectional molecular epidemiological study was carried out in the Autonomous Region of Andalusia (ARA, southern Spain) between 2012 and 2021. In this area, wild lagomorphs live in Iberian Mediterranean-scrubland ecosystems. Wild rabbits are organized in social groups of 5–13 animals and live in burrows whereas Iberian hares usually live alone, and they spend the day in depressions built by themselves. Both species feed mainly on grasses, and their peaks of activity are at sunset and to a lesser extent at night (MITECO, 2007; von Holst et al., 2002).

Whenever possible, 59 wild rabbits, the most important wild lagomorph species in terms of abundance and distribution in the Iberian Peninsula, were sampled in each of the eight provinces that form the study area to ensure a 95% probability of detecting a positive animal for an assumed minimum enteroparasite prevalence of 5% (Thrusfield & Christley, 2018). In addition, for each province, sampling sites (hunting states) were randomly selected. In each of these hunting estates, between 1 and 64 (mean: 7.7) animals were kindly provided by hunters to collect samples (Figure 1). A total of 439 wild rabbits legally harvested during the hunting season were sampled from 57 hunting estates distributed across the study region. In addition, 111 Iberian hares were collected in 64 hunting estates during the same study period and region using convenience sampling.

Harvested animals were sampled at the same location on each of the hunting estates where necropsy was carried out. Between 5 and 15 g of faeces were obtained directly from the rectum, transported to the laboratory under refrigeration and stored at −20°C until DNA/RNA extraction. All faecal samples were formed. Information about each animal, including location, year of sampling, age (according to bodyweight and body length; Morris, 1972) and sex, were

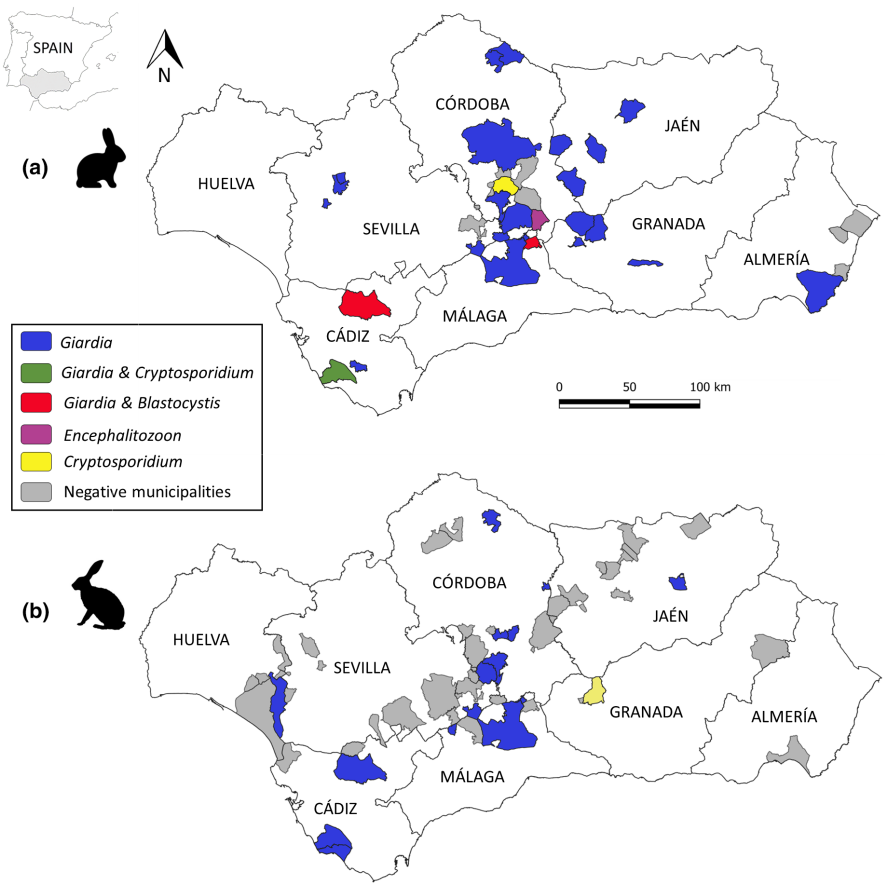

FIGURE 1 Map of the Autonomous Region of Andalusia (southern Spain) showing the sampling areas and the geographical distribution of the faecal samples from wild lagomorphs that tested positive for the protist and microsporidia species investigated in this study by municipality. Panel (a): wild rabbits. Panel (b): Iberian hares

recorded. Epidemiological data related to hunting estates were gathered through personal interviews with the gamekeepers using a standardized questionnaire. The information obtained included hunting estate's characteristics, disease control measures, management practices, and presence of other sympatric species (Table S1).

2.3 | DNA/RNA extraction and purification

Genomic nucleic acids were simultaneously isolated from about 100 mg of each individual faecal sample using the IndiSpin® Pathogen Kit (Indical Bioscience) according to the manufacturer's instructions. Extracted and purified DNA/RNA samples were eluted in 90 μl of PCR-grade water and kept at 4°C (DNA) or −80°C (RNA) until further molecular analysis.

2.4 | Molecular detection and characterization of *Giardia duodenalis*

Detection of *G. duodenalis* DNA was achieved using a real-time PCR (qPCR) method targeting a 62-bp region of the gene codifying the small subunit ribosomal RNA (*ssu* rRNA) of the parasite (Verweij et al., 2003). For assessing the molecular diversity of the parasite at the assemblage and sub-assemblage level, we adopted a sequence-based multilocus genotyping (MLST) scheme targeting the genes encoding for the glutamate dehydrogenase (*gdh*), β-giardin (*bg*), and triose phosphate isomerase (*tpi*) proteins of the parasite. Only samples that yielded qPCR C_T values <32 were assessed under the MLST scheme. A semi-nested PCR was used to amplify a 432-bp fragment of the *gdh* gene (Read et al., 2004), and nested PCRs were used to amplify 511- and 530-bp fragments of the *bg* and *tpi* genes, respectively (Lalle et al., 2005; Sulaiman et al., 2003).

2.5 | Molecular detection and characterization of *Cryptosporidium* spp.

The presence of *Cryptosporidium* spp. was assessed using a nested PCR protocol to amplify a 587-bp fragment of the *ssu* rRNA gene of the parasite (Tiangtip & Jongwutiwes, 2002). Subtyping of the isolates identified as *C. cuniculus* was carried out amplifying a 870-bp fragment of the *gp60* locus using a nested PCR (Feltus et al., 2006).

2.6 | Molecular detection of *Blastocystis* sp.

Identification of *Blastocystis* sp. was achieved by a direct PCR protocol targeting a 600-bp fragment of the *ssu* rRNA gene of the parasite (Scicluna et al., 2006).

2.7 | Molecular detection and characterization of *Enterocytozoon bieneusi*

Detection of *E. bieneusi* was conducted by a nested PCR protocol to amplify a 390-bp fragment of the internal transcribed spacer (ITS) region as well as portions of the flanking large and small subunit of the ribosomal RNA gene as described previously (Buckholt et al., 2002).

2.8 | Molecular detection of *Encephalitozoon* spp.

Initial detection of members of the genus *Encephalitozoon* was achieved using a generic direct PCR method targeting a 250–279-bp region of the *ssu* rRNA gene of parasites including *E. cuniculi*, *E. intestinalis*, and *E. hellem* (Fedorko et al., 1995).

2.9 | General PCR and electrophoretic procedures

Detailed information on the PCR cycling conditions and oligonucleotides used for the molecular identification and/or characterization of the protists parasites investigated in the present study is presented in Tables S2 and S3, respectively. All the direct, semi-nested, and nested PCR protocols described above were conducted on a 2720 Thermal Cycler (Applied Biosystems). Reaction mixes always included 2.5 units of MyTAQ™ DNA polymerase (Bioline GmbH), and 5–10 μl MyTAQ™ Reaction Buffer containing 5 mM dNTPs and 15 mM $MgCl_2$. Laboratory-confirmed positive and negative DNA samples of human and animal origin for each parasitic species investigated were routinely used as controls and included in each round of PCR. PCR amplicons were visualized on 1.5% D5 agarose gels (Conda) stained with Pronasafe (Conda) nucleic acid staining solutions. A 100-bp DNA ladder (Boehringer Mannheim GmbH) was used for the sizing of obtained amplicons.

2.10 | Sanger sequencing analyses

Positive PCR products of the expected size were directly sequenced in both directions using appropriate internal primer sets (Table S2). DNA sequencing was conducted by capillary electrophoresis using the BigDye® Terminator chemistry (Applied Biosystems) on an ABI PRISM 3130 automated DNA sequencer. Generated DNA consensus sequences were aligned to appropriate reference sequences using MEGA X (Kumar et al., 2018) for species confirmation and genotype identification. The sequences obtained in this study have been deposited in GenBank under accession numbers OP219713 (*G. duodenalis*), OP204216–OP204217 and OP219708–OP219712 (*Cryptosporidium* spp.), and OP204217 (*E. intestinalis*).

2.11 | *Blastocystis* subtype identification using next-generation amplicon sequencing

DNA aliquots of potential *Blastocystis*-positive samples by conventional *ssu*-PCR were shipped to the Environmental Microbial and Food Safety Laboratory, Agricultural Research Service, Beltsville, MD, (USA). A next-generation amplicon sequencing strategy and custom analysis pipeline were used to assess intra-isolate *Blastocystis* molecular diversity as previously described (Maloney, Molokin & Santín, 2019). Briefly, a PCR using the primer set ILMN_Blast505_532F and ILMN_Blast998_1017R (Table S2) was used to screen all positive/suspected samples by conventional *ssu*-PCR. These primers amplify a fragment of the *ssu* rRNA gene (ca. 500 bp) and are identical to Blast505_532F/Blast998_1017R (Santín et al., 2011), with the exception that they contain the Illumina overhang adapter sequences on the 5′ end. A final pooled library concentration of 8 pM with 20% PhiX control was sequenced using an Illumina MiSeq and 600 cycle, v3 chemistry (Illumina). Read pairs generated by the MiSeq were processed using an in-house pipeline that leveraged the following open-source Linux tools: BBTools package v38.82 (Bushnell, 2014), VSEARCH v2.15.1 (Rognes et al., 2016) and BLAST+ 2.11.0 (Camacho et al., 2009). Processing included the merging of read pairs, quality and length filtering, sequence denoising and chimera filtering. Then, filtered reads were clustered using a 98% identity threshold into operational taxonomic units (OTUs) within each sample. Any OTU with an abundance below 100 sequences was filtered out and the remaining OTUs were once more checked for chimeric sequences within each sample. OTUs were then aligned to *Blastocystis* references from NCBI using Blast+. An alignment length cut-off of 400 bp was used to filter out short blast hits. The nucleotide sequences generated using NGS in this study were deposited in GenBank under the accession numbers OP503622–OP503627.

2.12 | Statistical analyses

The prevalence of the different protist and microsporidia pathogens was determined from the proportion of positive samples to the total number of examined, using the two-sided exact binomial test, with 95% confidence intervals (95% CI). Continuous variables were categorized considering percentiles 33 and 66 as cut-off points. First, associations between results and explanatory variables were screened using the Pearson's Chi-square test or Fisher's test, as appropriate. All variables with a p-value <0.10 in the bivariate analysis were selected for further analyses. Collinearity between pairs of variables was tested by Cramer's V coefficient. Second, a generalized estimating equation (GEE) analysis was carried out to study the effect of the variables selected based on the bivariate analysis. The number of positive animals was assumed to follow a binomial distribution, and 'hunting estate' was included as the subject variable. The model was re-run until all remaining variables showed statistically significant values ($p < 0.05$). SPSS 22.0 software (IBM Corp.) was used for all statistical analyses.

3 | RESULTS

3.1 | Occurrence of microeukaryotic parasites

The full dataset showing the epidemiological, diagnostic and genotyping results generated in the present study is presented in Table S4. We collected faecal samples from wild lagomorphs in the eight provinces (Almería, Cádiz, Córdoba, Granada, Huelva, Jaén, Málaga and Sevilla) conforming the ARA. No rabbit faecal samples could be obtained from the Huelva province. All sampled leporids had no apparent gastrointestinal clinical manifestations, and the consistency of their excrements was formed.

Table 2 shows the PCR-based infection rates for the five microeukaryotic parasites investigated in the surveyed wild leporid populations. The most common pathogenic protozoa identified was *G. duodenalis* (27.8%, 153/550; 95% CI: 24.1–31.8), followed by *Cryptosporidium* spp. (1.3%, 7/550; 95% CI: 0.5–2.6), *Blastocystis* sp. (1.1%, 6/550; 95% CI: 0.4–2.4) and *E. intestinalis* (0.2%, 1/550; 95% CI: 0.0–10.1). Considering host species, we found *G. duodenalis* in 29.0% (127/438) of European wild rabbits and 22.5% (25/111) of Iberian hares. *Cryptosporidium* spp. was detected in faecal samples from 1.1% (5/438) wild rabbits and 1.8% (2/111) hares. *Blastocystis* sp. and *E. intestinalis* were identified in six and one wild rabbits, respectively, but not in hares. *Enterocytozoon bieneusi* DNA was not detected in any of the wild leporid faecal samples analysed. Co-infections involving two eukaryotic species were observed in six wild rabbit faecal samples. The combinations found were *G. duodenalis* + *C. andersoni* (16.7%; 1/6) and *G. duodenalis* + *Blastocystis* sp. (83.3%; 5/6; Figure 1).

Infections by enteric protists in wild rabbits showed geographical segregation patterns. Whereas *G. duodenalis* was present in all Andalusian provinces sampled (infection rates: 4%–42%), *Cryptosporidium* spp. infections were only detected in rabbits from Cádiz and Córdoba (infection rates: 2%–4%). *Blastocystis* sp. was present in single rabbit from Cádiz (infection rate: 4%; Figure 1). A similar pattern was observed in Iberian hares, where *G. duodenalis* was identified in 5 out of the 8 Andalusian provinces sampled (infection rates: 11%–43%) and *Cryptosporidium* spp. was detected at a 50% infection rate only in hares from the Granada province (Figure 1).

3.2 | Molecular characterization of *Giardia duodenalis* isolates

The 153 DNA isolates that yielded a positive result for *G. duodenalis* by qPCR generated cycle threshold (C_T) values ranging from 25.2 to 40.2 (median: 34.6). Genotyping analyses were only conducted in positive samples with C_T values ≤32 (14.4%, 22/153). We successfully amplified only one of them at the *gdh* (but not at the *bg* or *tpi*) locus corresponding to a European wild rabbit from Cádiz province (qPCR C_T value: 31.5). Sequence analyses revealed the presence of zoonotic sub-assemblage BIV. This sequence differed by six

TABLE 2 Distribution of infection rates of intestinal protist and microsporidial pathogens in wild lagomorphs in Andalusia (southern Spain)

Host species	No. of individuals	G. duodenalis *n* (%)	*Cryptosporidium* spp. *n* (%)	*Blastocystis* sp. *n* (%)	E. bieneusi *n* (%)	*Encephalitozoon* spp. *n* (%)
European wild rabbit						
Sex						
Males	221	74 (33.5)	0 (0.0)	4 (1.8)	0 (0.0)	0 (0.0)
Females	214	53 (24.8)	5 (2.3)	2 (0.9)	0 (0.0)	1 (0.5)
Missing values	4	1 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Age						
Adults	213	68 (31.9)	0 (0.0)	5 (2.3)	0 (0.0)	0 (0.0)
Sub-adults	118	38 (32.2)	0 (0.0)	1 (0.9)	0 (0.0)	0 (0.0)
Juveniles	39	12 (30.8)	1 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)
Missing values	69	10 (14.5)	4 (5.8)	0 (0.0)	0 (0.0)	1 (1.5)
Sampling period						
2012/13[a]	64	8 (12.5)	4 (6.2)	0 (0.0)	0 (0.0)	0 (0.0)
2018/19	0	–	–	–	–	–
2019/20	7	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
2020/21	368	119 (32.3)	1 (0.3)	6 (1.6)	0 (0.0)	1 (0.3)
Missing values	0	–	–	–	–	–
Geographic origin						
Almería	55	3 (5.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cádiz	110	42 (38.2)	4 (3.6)	4 (3.6)	0 (0.0)	0 (0.0)
Córdoba	52	22 (42.3)	1 (1.9)	0 (0.0)	0 (0.0)	1 (1.9)
Granada	60	14 (23.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Huelva	0	–	–	–	–	–
Jaén	56	16 (28.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Málaga	53	16 (30.2)	0 (0.0)	2 (3.8)	0 (0.0)	0 (0.0)
Sevilla	53	15 (28.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Missing values	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Subtotal	439	128 (29.2)	5 (1.1)	6 (1.4)	0 (0.0)	1 (0.2)
Iberian hare						
Sex						
Males	53	10 (18.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Females	57	14 (24.6)	2 (3.5)	0 (0.0)	0 (0.0)	0 (0.0)
Missing values	1	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Age						
Adults	90	21 (23.3)	2 (2.2)	0 (0.0)	0 (0.0)	0 (0.0)
Sub-adults	15	3 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Juveniles	5	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Missing values	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Sampling period				0 (0.0)	0 (0.0)	0 (0.0)
2012/13[a]	0	–	–	–	–	–
2018/19	33	1 (3.0)	2 (6.1)	0 (0.0)	0 (0.0)	0 (0.0)
2019/20	7	2 (28.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
2020/21	71	22 (31.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Missing values	0	–	–	–	–	–

(Continues)

TABLE 2 (Continued)

Host species	No. of individuals	G. duodenalis *n* (%)	*Cryptosporidium* spp. *n* (%)	*Blastocystis* sp. *n* (%)	E. bieneusi *n* (%)	*Encephalitozoon* spp. *n* (%)
Geographic origin						
Almería	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cádiz	7	3 (42.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Córdoba	33	12 (36.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Granada	4	0 (0.0)	2 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)
Huelva	9	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Jaén	16	2 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Málaga	19	7 (36.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Sevilla	21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Missing values	0	–	–	–	–	–
Subtotal	111	25 (22.5)	2 (1.8)	0 (0.0)	0 (0.0)	0 (0.0)
Total	550	153 (27.8)	7 (1.3)	6 (1.1)	0 (0.0)	1 (0.2)[b]

[a]No associated epidemiological data available for samples collected in the 2012/13 sampling period.
[b]*Encephalitozoon intestinalis*.

single-nucleotide polymorphisms (SNPs) with reference sequence L40508 (Table 3).

3.3 | Molecular characterization of *Cryptosporidium* spp. isolates

All seven *Cryptosporidium*-positive samples by *ssu*-PCR were successfully sequenced, allowing the identification of two *Cryptosporidium* species including leporid-adapted *C. cuniculus* (85.7%; 6/7) and bovine-adapted *C. andersoni* (14.3%; 1/7). *Cryptosporidium cuniculus* was identified in four rabbits (three from Cádiz and one from Córdoba provinces) and two hares (both from Granada province), whereas *C. andersoni* was found in a rabbit from Cádiz province (Figure 1). All six *C. cuniculus* sequences generated at the *ssu* rRNA locus were identical and showed 100% identity with reference nucleotide sequence EU437413. Genotyping PCRs at the *gp60* marker were successful for five of them, revealing the presence of subtypes VaA16 ($n = 1$), VaA18 ($n = 2$), VbA24 ($n = 1$), and VbA31 ($n = 1$). The obtained *ssu* rRNA *C. andersoni* sequence differed by a single SNP from reference sequence AB513856 (Table 3).

3.4 | Molecular characterization of *Blastocystis* sp. isolates

Six faecal samples of wild rabbits from the Cádiz ($n = 4$) and Málaga ($n = 2$) provinces yielded amplicons of the expected size for *Blastocystis* sp. on agarose gels. Sanger sequencing of these PCR products was unsuccessful. They were, therefore, reanalysed by next-generation amplicon sequencing. These analyses revealed the presence of *Blastocystis* ST2 in one wild rabbit from the Cádiz and two wild rabbits from Málaga provinces. Two genetic variants of ST2 were observed in the ST2-infected wild rabbit from the Cádiz province. Additionally, three wild rabbit samples from the Cádiz province carried ST1 + ST2 mixed infections.

3.5 | Molecular characterization of *Encephalitozoon* spp. isolates

Encephalitozoon intestinalis was detected in a single wild rabbit faecal sample from the Córdoba province (Figure 1). Obtained nucleotide sequence showed 100% identity with reference sequence EU436735 (Table 3). A second wild rabbit faecal sample from the Seville province yielded a band of the expected size on agarose gel, but lack of confirmation by Sanger sequencing precluded us to consider this as a true Microsporidia-positive result (Table S4).

3.6 | Statistical analyses

Leporids infected by *Cryptosporidium* spp., *Blastocystis* sp. and *E. intestinalis* were not included in the analyses due to the low number of positive samples and insufficient statistical power. Results of the bivariate analysis for assessing the distribution of *G. duodenalis* infections in wild rabbits and hares according to the variables considered in the present study are shown in Table S5. GEE analyses showed that geographical area and weeding were potential risk and protective factors, respectively, associated with *Giardia* infections in wild lagomorphs in the study area. Significantly higher prevalence of this parasite was observed in leporids from Central Andalusia ($p = 0.001$; OR = 3.4; 95% CI: 1.6–7.0) compared with Eastern areas and in leporids from hunting estates that do not carry out weed

TABLE 3 Frequency and molecular diversity of *Giardia duodenalis*, *Cryptosporidium* spp., *Blastocystis* sp. and *Encephalitozoon intestinalis* identified in wild lagomorphs, Andalusia (southern Spain) 2012–2021

Species	Sub-genotype	No. of isolates	Locus	Reference sequence	Stretch	Single-nucleotide polymorphisms	GenBank ID
G. duodenalis	BIV	1	*gdh*	L40508	76–496	A230G, A287G, T344G, T387C, T431Y, C468T	OP219713
C. andersoni	ND	1	*ssu* rRNA	AB513856	475–979	G943A	OP204216
C. cuniculus	–	6	*ssu* rRNA	EU437413	311–787	None	OP204217
	VaA16	1	*gp60*	KU852733	1–775	61_69delTCATCATCA, 125_127delAAC	OP219708
	VaA18	1	*gp60*	FJ262730	9–855	A490G	OP219709
	VaA18	1	*gp60*	FJ262730	14–855	A490G, G823T	OP219710
	VbA24	1	*gp60*	MG516797	7–773	85_90delTCATCA	OP219711
	VbA31	1	*gp60*	KU852732	10–802	None	OP219712
Blastocystis sp.[a,b]	ST2	4	*ssu* rRNA	–	–	–	OP503622
							OP503623
		2					OP503626
		1					
	ST1	1	*ssu* rRNA	–	–	–	OP503624
		1					OP503625
		1					OP503627
E. intestinalis	NA	1	*ssu* rRNA	EU436735	56–271	None	OP204218

Abbreviations: del, Deletion; *gdh*, glutamate dehydrogenase; *gp60*, 60Kda glycoprotein; NA, not applicable; ND, not done; *ssu* rRNA, small subunit ribosomal RNA.

[a]Subtypes identified by next-generation amplicon sequencing using Illumina technology.

[b]One sample contained two genetic variants of ST2 and three samples contained ST1 and ST2.

TABLE 4 Generalized estimating equations analysis of risk factors associated with *Giardia duodenalis* infection in wild lagomorphs in Andalusia (southern Spain)

Variable	Category	*p*-value	OR (95% CI)[a]
Geographical area	Western	0.161	2.0 (0.8–5.2)
	Central	0.001	3.4 (1.6–7.0)
	Eastern	Reference	Reference
The hunting estate is weeded	No	0.028	4.6 (1.2–17.8)
	Yes	Reference	Reference

[a]95% CI, 95% confidence intervals.

management ($p = 0.028$; OR = 4.6; 95% CI: 1.2–17.8) compared with weeded states (Table 4).

4 | DISCUSSION

The epidemiology of intestinal eukaryotes in wild leporids is poorly understood, particularly in European countries including Spain. Leporids infected with diarrhoea-causing microeukaryotes might contribute to the environmental burden of cysts/oocysts/spores and contaminate soils, pastures, and surface waters intended for livestock and/or human consumption. As an example of their public health relevance, a waterborne outbreak of *C. cuniculus* VaA18 caused by an infected wild rabbit carcass in contact with the potable water supply was reported in Northamptonshire, UK (Puleston et al., 2014). The present study provides new epidemiological data on the occurrence, genetic diversity and zoonotic potential of the enteric microeukaryotic species of main public health significance in wild leporid populations in Mediterranean ecosystems of southern Spain. The major strengths of the survey include a large sample size, which is representative of the whole study area, the use of highly sensitive PCR methods coupled with Sanger and next-generation amplicon sequencing for detection and genotyping purposes and the availability of a full dataset associated to the samples collected that allowed consistent statistical analyses.

We detected the presence of *G. duodenalis* in 29% of wild rabbits and 23% of hares, this being the first report of the parasite in these host species in Spain. There are, however, three previous Spanish studies detecting *G. duodenalis* in phylogenetically closely related members of the Rodentia order: prevalence rates of 14–38% have been described in black (*Rattus rattus*) and/or brown (*Rattus norvegicus*) rats in the Canary Islands (Fernández-Álvarez

et al., 2014), Catalonia (Galán-Puchades et al., 2021) and Córdoba (Köster et al., 2021), and in 18% of house mice (*Mus musculus*) in the Canary Islands (Fernández-Álvarez et al., 2014). Although most *G. duodenalis* isolates identified in the latter survey were assigned to rodent-specific assemblage G, a rat isolate was identified as zoonotic sub-assemblage BIV (Fernández-Álvarez et al., 2014), the same genetic variant identified in the only wild rabbit sample genotyped in the present study. In the European scenario, a *G. duodenalis* infection rate of 8% (40/528) was documented by coproantigen ELISA in pet rabbits mostly from Germany but positive results were also reported from Austria, Denmark, Hungary, Finland, France, Italy, Luxembourg, the Netherlands, Norway, Poland, Portugal, and Sweden (Pantchev et al., 2014). As in the case of our study, only one out of the 40 *Giardia*-positive samples was successfully genotyped as assemblage B at the *gdh* locus, but not at the *bg* and *tpi* loci (Pantchev et al., 2014). Assemblage B has also been sporadically identified in Swedish (Lebbad et al., 2010, 2011) and Chinese (Sulaiman et al., 2003) rabbit isolates used in genotyping or diagnostic surveys, suggesting that domestic (and very likely wild) rabbits are primarily infected by this zoonotic genetic variant of *G. duodenalis*. It may also be important to consider new strategies for assessing the assemblages within *Giardia*-positive samples given that sequence data are needed to determine the zoonotic potential of positive animals.

In the present study, positive animals to *G. duodenalis* were detected in every analysed hunting season and province of the study area. A significantly higher prevalence of the parasite was found in Central Andalusia. These results suggest that *G. duodenalis* is endemic in wild leporids in southern Spain, although the distribution of the infection is not homogeneous among and within provinces. In addition, GEE analysis identified that lagomorphs inhabiting weeded hunting estates were at lower risk for *G. duodenalis* infection. These findings seem to indicate that environmental (e.g. climate and humidity) and management (e.g. land clearing and presence of livestock) factors may influence the persistence of the parasite and its transmission success. More research should be conducted to investigate how and to which extent environmental factors contribute to the spreading of *G. duodenalis* in Spanish Mediterranean ecosystems.

In this study, *Cryptosporidium* infections were identified in 1% of wild rabbits and 2% of hares. These findings were similar to that (4%, 4/100) reported recently in wild rabbits in Tenerife (Canary Islands, Spain) where the only isolate characterized belonged to *C. cuniculus* subtype VbA26R3 (Baz-González et al., 2022). This result agrees with our molecular findings showing that *Cryptosporidium*-positive samples belonged to *C. cuniculus gp60* subtypes in families Va and Vb. Information on *Cryptosporidium* infections in European rabbits is very scarce: in a seminal study conducted in the Czech Republic, *C. cuniculus* (formerly *Cryptosporidium* rabbit genotype; Robinson et al., 2010) was identified in two farmed rabbits with unspecified clinical status (Ryan et al., 2003). A fatal diarrhoea outbreak by *C. cuniculus* VbA24 has been also documented in farmed rabbits in Poland (Kaupke et al., 2014). The detection of cattle-adapted *C. andersoni* in a wild rabbit from Cádiz province is relevant, as it suggests potential cross-species transmission in areas where cattle and rabbits live sympatrically. However, we cannot rule out the possibility that this finding is the result of a spurious (mechanical carriage) infection. Previous studies have identified sporadic infections by *C. andersoni* wildlife such as red foxes (Kvac et al., 2021) and small rodents (Vioque et al., 2022).

The stramenopile *Blastocystis* sp. is a microeukaryote of uncertain pathogenicity (Tan et al., 2010). *Blastocystis* infection/carriage is particularly common in strict herbivorous mammals, mainly livestock. High prevalence rates (Hublin et al., 2021; Shams et al., 2021) and concomitant infections by several *Blastocystis* subtypes have been reported globally in cattle (Abarca et al., 2021; Maloney, Lombard, et al., 2019). In sharp contrast, *Blastocystis* sp. seems far less frequent in wild and domestic leporids: infection/carriage rates of 1%–3% have been reported in farmed New Zealand white rabbits (*O. cuniculus*) and tolai hares (*Lepus tolai*) in China (Li et al., 2020; Wang et al., 2018). All the *Blastocystis*-positive samples identified in those Chinese studies were genotyped as ST4. Additionally, *Blastocystis* ST14 has been reported in a single pet rabbit isolate in the United Arab Emirates (AbuOdeh et al., 2019). The finding of ST2, individually or in combination with ST1, in the present study is relevant because they represent the first description of these potentially zoonotic subtypes in lagomorphs globally, expanding our current knowledge on the epidemiology and host range of *Blastocystis* sp.

Leporids including rabbits and hares are recognized as suitable hosts for microsporidial pathogens, particularly *E. cuniculi* and, to a lesser extent, *E. bieneusi* (Mathis et al., 2005; Wasson & Peper, 2000). Although *E. cuniculi* usually causes subclinical infection in humans, it can also lead to encephalitozoonosis, a clinical disease characterized by neurological, ocular and/or renal signs that can be even fatal, especially in immunocompromised individuals (Dipineto et al., 2008; Künzel et al., 2008; Magalhães et al., 2022; Valencakova et al., 2008). In a seminal Spanish study, *E. bieneusi* was described for the first time in wild rabbits (del Aguila et al., 1999). *Enterocytozoon bieneusi* genotype D has also been identified in a farmed rabbit isolate (Galván-Díaz et al., 2014). Of note, *E. bieneusi* and *E. intestinalis* (but not *E. hellem* or *E. cuniculi*) were detected in kidney and/or brain tissues of wild European rabbits and Iberian hares in southern Spain, suggesting that meat consumption of infected animals can be of public health concern (Martínez-Padilla et al., 2020). More recently, the critically endangered Iberian lynx has been shown to be infected by microsporidial pathogens by molecular and serological methods in southern Spain (Izquierdo et al., 2022). Because rabbits are the staple prey for lynx in this region, these findings suggest that microsporidia-infected rabbits could pose a risk for Iberian lynx conservation. *Encephalitozoon cuniculi* antigens were also detected in 18% (4/22) of faecal samples from domestic rabbits in northwestern Spain using anti-*E. cuniculi* serum (Lores et al., 2002).

However, it should be noted that serology reflects contact with the pathogen, but not necessarily infection. Another potential limitation of serological test is the insufficiently tested cross-reactivity with phylogenetically related microorganisms, which can lead to false-positive results. For this reason, molecular-based surveys are

necessary to disentangle the epidemiology of microsporidial pathogens including *E. bieneusi* and *Encephalitozoon* spp.

A major limitation of this study is the relatively low amplification success rate achieved by the PCR protocols used for genotyping purposes. The most likely explanation for this issue is the combined effect of (i) light parasite burdens (as suggested by the predominance of formed, non-diarrhoeic faecal samples and, in the case of *G. duodenalis*, high C_T values at qPCR), and (ii) the intrinsic limited sensitivity of the single-copy genes (*Giardia*: *gdh*, *bg* and *tpi*; *Cryptosporidium*: *gp60*) targeted in the above-mentioned PCR protocols. Long-term preservation of some faecal samples might have altered the quality/quantity of parasitic DNA and biased our prevalence or genotyping results. Finally, selected sampling areas might not be fully representative of the geographical distribution of the microeukaryotic species investigated, leading to misidentification of pockets of infection and underestimation of true prevalences. Care should be taken when attempting to extrapolate these data to other Spanish regions.

5 | CONCLUSIONS

This is the largest and most thorough molecular-based epidemiological study aiming at investigating the occurrence, genetic diversity and zoonotic potential of microeukaryotic enteroparasites in wild leporids conducted in Europe to date. Our molecular data demonstrate that wild rabbits (and likely Iberian hares also) are suitable hosts for zoonotic species/genotypes including *G. duodenalis* subassemblage BIV, *C. cuniculus*, *Blastocystis* ST1 and ST2, and *E. intestinalis*. Hunters, farmers and individuals in close contact with wild rabbits and hares (or their excrements) should be regarded as at risk of infection by the above-mentioned pathogenic enteroparasites. The identification of cattle-adapted *C. andersoni* is indicative of an overlap between domestic/peri-domestic and wild transmission cycles of this agent. Results generated in the present survey should be corroborated in other Spanish regions for which this information is not currently available.

AUTHOR CONTRIBUTIONS

SCS, DJM, JCG, DCT and IGB collected the samples. LR, CC, PCK, BB, AD, JGM, CHC and FV carried out the laboratory experiments. PCK, AD and JGM conducted sequence analyses. DCT conducted statistical analyses. MS, IGB, DC and DGB designed and supervised the experiments. AD, DGB and DC writing—original draft preparation. DCT, JGM, MS, IGB, DGB and DC writing—review and editing. The final version was read and approved by all authors.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available within the main body of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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