Ticks and Tick-borne Diseases 10 (2019) 848–852

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

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

Original article

Endosymbionts carried by ticks feeding on dogs in Spain

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ARTICLE INFO

Keywords:
'*Candidatus* Rickettsiella isopodorum'
Rickettsia
Coxiella
Francisella
Tick-borne pathogens

ABSTRACT

Studies on tick microbial communities historically focused on tick-borne pathogens. However, there is an increasing interest in capturing relationships among non-pathogenic endosymbionts and exploring their relevance for tick biology. The present study included a total of 1600 adult ticks collected from domestic dogs in 4 different biogeographical regions of Spain. Each pool formed by 1 to 10 halves of individuals representing one specific ticks species was examined by PCR for the presence of *Coxiellaceae, Rickettsia* spp., *Rickettsiales*, *Wolbachia* spp., and other bacterial DNA. Of the pools analyzed, 92% tested positive for endosymbiont-derived DNA. *Coxiella* spp. endosymbionts were the most prevalent microorganisms, being always present in *Rhipicephalus sanguineus* sensu lato (s.l.) pools. *Rickettsia* spp. DNA was detected in 60% of *Dermacentor reticulatus* pools and 40% of *R. sanguineus* s.l. pools, with a higher diversity of *Rickettsia* species in *R. sanguineus* s.l. pools. Our study reveals a negative relationship of *Rickettsia massiliae* with the presence of tick-borne pathogens in the same pool of ticks. An additional endosymbiont, '*Candidatus* Rickettsiella isopodorum', was only detected in *D. reticulatus* pools. Data from this study indicate that dogs in Spain are exposed to several endosymbionts. Due to the importance of tick-borne pathogens, characterizing the role of endosymbionts for tick physiology and prevalence, may lead to novel control strategies.

1. Introduction

Ticks (Acarina) are among the most prominent arthropod vectors of pathogens to humans and domestic animals worldwide, transmitting the largest range of viruses, parasites, and bacteria (Estrada-Peña et al., 2015; Moutailler et al., 2016). Tick-borne diseases have a large and growing social and economic impact (Michelet et al., 2016). The current trends of climate and the unpredictability of long-term changes, towards warmer and shorter autumn and winters, together with changes in human social habits and human-derived actions on the landscape, deeply modifying natural habitats, have raised concerns about the (re)emergence of tick-borne diseases and their geographical spread (Michelet et al., 2016; Papa et al., 2017).

Spain is a western Mediterranean country where several tick species and tick-borne pathogens have been reported (Estrada-Peña et al., 2017). A recent study reported the main species of ticks feeding on owned dogs, including *Rhipicephalus sanguineus* sensu lato (s.l.) (53% of all collected ticks), *Dermacentor reticulatus* (9%), *Ixodes ricinus* (9%), and *Ixodes hexagonus* (4%) (Estrada-Peña et al., 2017). Previous studies reported the prevalence of tick-borne pathogens in dogs in Spain, where *Ehrlichia canis* (5–54.7%), *Anaplasma* spp. (3.1–45.3%), *Rickettsia conorii* (24.6–50%), and *Borrelia burgdorferi* sensu lato (s.l.) (6.3–8.8%) were the most commonly reported pathogens with rates of infection varying across the geography (Amusátegui et al., 2008; Miró et al., 2013).

Over the last few decades, considerable research efforts have focused on the diversity, composition, and effects of tick microbial communities on either tick physiology or the coexistence with pathogens. These communities include non-pathogenic microorganisms such as commensal and mutualistic microbes called endosymbionts (Bonnet et al., 2017; Moutailler et al., 2016; Taylor et al., 2012). The best known examples are found within members of the genera *Rickettsia*, *Francisella*, and *Coxiella* (Ahantarig et al., 2013; Bonnet et al., 2017). The impact of endosymbionts on the tick, its vertebrate host and other tick microbiota, remains largely uninvestigated (Bonnet et al., 2017). Nonetheless, endosymbionts can have multiple effects (detrimental or beneficial) on their carrier tick, playing a role in fitness, adaptation, development, reproduction or immunity (Ahantarig et al., 2013; Gray

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https://doi.org/10.1016/j.ttbdis.2019.04.003
Received 19 September 2018; Received in revised form 25 March 2019; Accepted 4 April 2019
Available online 06 April 2019
1877-959X/

Table 1
Primers used for each specific PCR amplification. The amplified region, the sequence of both primers, and the final primer concentration in the PCR mix are shown.

Microorganism	Region amplified	Primer Forward (5'-3')	Primer Reverse (5'-3')	(μM)
Rickettsiales	16S rRNA	GCAAGCYTAACACATGCAAGTCG	CTACTAGGTAGATTCCTAYGCATTACTCACC	0.5
Coxiellaceae	16S rRNA	TTTCGGTGGGGAAGAAATTCTC	ACTTAAATATCCACCTACGCGCG	0.3
***Rickettsia* spp.**	ITS2	GCTCGATTGRTTTACTTTGCTGTGAG	CATGCTATAACCACCAAGCTAGCAATAC	0.5/0.3
***Wolbachia* spp.**	16S rRNA	GGCAACTAATACCGTATACGCCCTA	GTATCTCAGTTCCAGTGTGGCTGA	0.3
Bacterial	16S rRNA	GCAAGCYTAACACATGCAAGTCG	TGCTGCCTCCCGTAGGAGT	0.3
	16S rRNA	AGAGTTTGATCCTGGCTCAG	CTACTAGGTAGATTCCTAYGCATTACTCACC	0.5/0.3

et al., 2009; Guizzo et al., 2017; Papa et al., 2017). Each endosymbiont may have a different role depending on the tick species (Ahantarig et al., 2013; Kurlovs et al., 2014; Moutailler et al., 2016), and ticks may host a mixture of endosymbionts and/or be co-infected with potential pathogens (Ahantarig et al., 2013). Moreover, endosymbionts may influence the transmission of pathogens to the vertebrate host. For example, rickettsial endosymbionts are thought to alter transmission of rickettsial pathogens (Moutailler et al., 2016). It has been stated that the presence of other rickettsial-like endosymbionts such as *Coxiella* in the salivary glands of *Amblyomma* ticks may impair the transmission of *Ehrlichia chaffeensis* (Bonnet et al., 2017; Estrada-Peña et al., 2015). However, the exact role of endosymbionts of ticks in the transmission of pathogens remains unexplored.

This study builds on previous studies of the tick fauna of owned dogs in Spain. We examined a large number of adults of four species of ticks (*R. sanguineus* s.l., *D. reticulatus, I. ricinus,* and *I. hexagonus*), collected feeding on dogs at a variety of sites and covering every biogeographical region in Spain (Estrada-Peña et al., 2017), with the primary aim to identify their rickettsial or rickettsial-like endosymbionts, and describing their prevalence. A secondary aim was to detect co-infections between the endosymbionts and bacterial or protozoan pathogens in the ticks, acknowledging the limitations emanating from the procedures outlined above.

2. Material & methods

2.1. Sample collection

A total of 1600 ticks collected during a previous study performed in Spain by Estrada-Peña et al. (2017), were analyzed. Ticks were collected from owned dogs to evaluate the presence of endosymbionts in specific tick species. The veterinarians, obtaining written consent for the collection of the ticks, informed dog owners about the protocol. Ticks attached to the dogs were carefully removed by veterinarians using fine tweezers and stored in high-quality 70% ethanol, and submitted to the researchers. Adults of four species of ticks in 4 different genera were assayed: *R. sanguineus* s.l., *D. reticulatus*, *I. ricinus*, and *I. hexagonus*. Every tick was classified to species level and identity checked twice by a specialist in tick taxonomy. No *I. inopinatus* was collected on dogs, and only *R. sanguineus* s.l. ticks were identified to "group" level since the re-description of *R. sanguineus* s.s. (Nava et al., 2018) was not yet available at that time. Ticks were pooled by tick species and by biogeographical region of origin (summarized as Northwest, North, Center, Mediterranean, and South). These regions represent a rough ecological division of the territory, in which a clear differential distribution of tick species has been demonstrated. *Rhipicephalus sanguineus* s.l. is the only species found in Center, Mediterranean, and South, while variable proportions of other species coexist with them in North and Northwest (Estrada-Peña et al., 2017). A total of 418 pools (region/month/species) of 1 to 6 ticks were included. Collection protocols, identification of ticks, and geographical analysis have been previously described (Estrada-Peña et al., 2017).

2.2. DNA extraction

DNA extraction was performed using High Pure PCR Template preparation kit (Roche®, Pleasanton, USA) according to the manufacturer's instructions with some modifications. Each pool was washed in sterile phosphate-buffered saline (PBS) solution overnight at 4 °C to eliminate residual alcohol. The day after, once PBS was eliminated, every pool of ticks was mechanically crushed using a Schwingmühle Tissue Lyser II Retsch (Qiagen®, Hilden, Germany) with one stainless steel bead of 5 mm in the lysis buffer of the kit. A piece of spleen of a healthy dog was used as a control of extraction to ensure that no cross-contamination occurred during DNA extraction.

2.3. Endosymbiont detection

The presence of *Coxiellaceae, Rickettsia* spp., *Rickettsiales, Wolbachia* spp., and other bacterial DNA in tick extracts was tested by PCR using specific primers for each of these groups. The primer sets used are listed in Table 1.

Real time PCR was carried out in a final volume of 20 μl using SYBR SELECT master mix (AB, Life technologies®, Carlsbad, USA), 4 μl of diluted DNA (dilution 1/2) and the corresponding primer for each one of the PCR assay. Primers designed by Vetgenomics (www.vetgenomics.com) (2018, June) were used for amplification. PCR were performed in 7900 H T or QuantStudio 7 Flex real time equipment. The thermal cycling profile was 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min and a dissociation curve added at the end of the run. The amplified region, the sequence of both primers, and the final primer concentration in the PCR are shown in Table 1. The eukaryotic 18S RNA Pre-Developed TaqMan assay (ThermoFisher®, Waltham, USA) using the same method detailed in Estrada-Peña et al. (2017), was used as an internal reference for tick genomic DNA amplification to ensure the proper PCR amplification of each pool so that negative results corresponded to true negative pool rather than a problem with DNA loading, sample degradation or PCR inhibition. Positive pools were sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (AB, Life technologies®) using the same primers. Sequences obtained were compared with GenBank (www.ncbi.nlm.nih.gov/BLAST) (2018, September) and Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) (2018, September).

2.4. Pathogen detection

The same pools of ticks had already been screened for the presence

of tick-borne pathogens as part of a previous study (Estrada-Peña et al., 2017). Of the 418 analyzed pools, 98 (23.4%) were positive for tick-borne pathogens. Among them, 63 were positive for *Babesia canis, B. gibsoni* or *B. vogeli*; 31 were positive for *Anaplasma platys* or *A. phagocytophilum*; and 8 were positive for *Theileria* spp. Other pathogens found were *Cytauxzoon felis, Hepatozoon canis,* and *Ehrlichia canis.*

2.5. Statistical analysis

The differences between the presences of different endosymbiont species were tested for significance by chi-squared analysis or Fisher's exact test, using GraphPad Software (http://graphpad.com/quickcalcs/contingency2/). A $P < 0.05$ was regarded as statistically significant.

3. Results

A total of 92% (384/418) of the examined tick pools were positive for endosymbiont-derived DNA.

A *Coxiella* sp. (*R. sanguineus* s.l. symbiont) identical to a sequence reported with a GenBank accession number D84559, was always present in the 315 pools of *R. sanguineus* s.l., in every biogeographical region (Table 2). Detection rates for this *Coxiella* sp. were lower for *I. ricinus* 12% (4/32) and *I. hexagonus* 4% (1/25), and it was absent from *D. reticulatus* and *H. marginatum*. We recorded another *Coxiella* sp. only in *Ixodes* ticks, which was identical to sequences reported with a GenBank accession numbers KP994817-18, KP994823-26 and KJ459074. It was found in all pools of *I. hexagonus* and in 6% (2/32) of *I. ricinus* pools.

The second most commonly detected endosymbionts were *Rickettsia* spp. with 154 out of 418 pools (36.8%) being positive. Details about species of endosymbionts associated with every species of tick and the number of positive tick pools are shown in Table 2. *Rickettsia* spp. DNA was detected in 40% (126/315) of *R. sanguineus* s.l. pools and 60% (26/43) of *D. reticulatus* pools. Two pools of *I. ricinus* (one in North, the other in South) carried *Rickettsia massiliae* previously described with GenBank accession numbers CP000683 and CP003319, and *Rickettsia monacensis* identical to a sequence reported with the GenBank accession number MG450331, respectively. No *Rickettsia* spp. DNA was detected in *I. hexagonus* or *H. marginatum. Rhipicephalus sanguineus* s.l. presented the highest diversity of *Rickettsia* species. *Rickettsia massiliae* was detected in about 37% (116/315) pools of *R. sanguineus* s.l., of which 67% (78/116) were identical to the strains G83, AZT80 and Bar29. Furthermore, *Rickettsia sibirica mongolitimonae* previously reported with GenBank accession numbers HQ710799, HQ710800 and DQ821875 was detected in 7 pools (9.8%) of *R. sanguineus* s.l. from Center. Other findings were *R. monacensis* in 2 pools (3.3%) from South, and a DNA compatible with known sequences of *Rickettsia raoultii* identical to sequences reported with GenBank accession numbers MG450327 and KX161769 in 1 pool from the North. Sequences representing *R. raoultii* were found in 60% (26/43) of *D. reticulatus* pools. The specimens of *D. reticulatus* were also positive for '*Candidatus* Rickettsiella isopodorum' displaying a 100% of identity with the new lineage of isopod-associated *Rickettsiella* bacteria previously reported with a GenBank accession number JX406180.

A *'Candidatus* symbiont' identical to sequences reported with the GenBank accession numbers DQ788562, JQ031634, CP002130 and AJ566640 was found only in the pools of *I. ricinus*, with a total prevalence of 12% (4/32 pools). The sequence found was 92% similar to '*Candidatus* Nicolleia massiliensis', a new genus from the *Rickettsiales* detected in *I. ricinus,* and 90% with '*Candidatus* Midichloria mitochondrii'. This endosymbiont was recorded in 7% (2/27 pools) and 40% (2/5 pools) of the pools of *I. ricinus* from North and Northwest, whereas the only specimen from South yielded negative results.

Finally, another sequence recorded in 6 out of 43 pools (13%) of *D. reticulatus* had 99% identity with previously described sequences. The high identity was found with some *Francisella*-like endosymbionts associated with GenBank accession numbers CP009654, MG859281, KX852465, JX561116, MH 329652, HQ705173 and EU234535 described in French, Bulgarian and Eurasian ticks, led us to classify this sequence as *Francisella*-like endosymbiont.

The relationships between all these endosymbionts and the previously reported pathogens (*Babesia* spp., *Anaplasma* spp., *Theileria* spp., *C. felis, H. canis,* and *E. canis.*) were calculated for 74/418 of positive pools (17.7%). The detection of *R. massiliae* in pools was related with the absence of the above pathogens in the same pool of *R. sanguineus* s.l. Other bacteria were detected only in 18/74 (15%) of the *R. sanguineus* s.l. pools positive for *R. massiliae*, while *R. massiliae* was absent in 56/74 pools (27%) of *R. sanguineus* s.l. that were positive for pathogens ($P = 0.0091$).

4. Discussion

This study recorded a high prevalence of endosymbionts, including *Coxiella* spp. and *Rickettsia* spp. in *R. sanguineus* s.l., *I. ricinus*, *I. hexagonus*, and *D. reticulatus* feeding on owned dogs in Spain. Two other

Table 2
Endosymbionts found in each pool of ticks.

Region	Center	Northwest	Mediterranean	North	South	Total
Number of total pools	**71**	**61**	**124**	**98**	**64**	**418**
***Rhipicephalus sanguineus* group**	**71**	**33**	**123**	**27**	**61**	**315**
Coxiella sp. (*Rhipicephalus sanguineus* symbiont)	71	33	123	27	61	315
Rickettsia massiliae	14	29	61	6	6	116
Rickettsia sibirica subsp. *mongolitimonae*	7					7
Rickettsia monacensis					2	2
Rickettsia raoultii				1		1
Dermacentor reticulatus		**12**	**1**	**30**		**43**
Francisella-like endosymbiont		2	1	3		6
'*Candidatus* Rickettsiella isopodorum'		1		1		2
Rickettsia raoultii		4		22		26
Ixodes ricinus		**5**		**27**	**1**	**33**
'*Candidatus* symbiont'		2		2		4
Rickettsia massiliae				1		1
Coxiella sp. (*Ixodes* symbiont)		2				2
Coxiella sp. (*Rhipicephalus sanguineus* symbiont)				4		4
Rickettsia monacensis					1	1
Ixodes hexagonus		**11**		**14**	**1**	**26**
Coxiella sp. (*Ixodes* symbiont)		11		14		25
Coxiella sp. (*Rhipicephalus sanguineus* symbiont)					1	1
Hyalomma marginatum					1	1

symbionts are currently known as '*Candidatus* symbiont' and *Francisella*-like, and no additional information is available about their specific status. The study has obvious limitations mainly related to the processing of ticks, since the raw material was already stored in pools, which renders specific combinations of pathogens-microbiome hard to compare with other reports, in which ticks were examined individually, specimens were collected in different biogeographical regions, or other tick stages were analyzed. The results are nevertheless suggestive of specific combinations of rickettsial symbionts and pathogenic microorganisms.

Our results showed a higher prevalence of endosymbionts in the studied pools of ticks (92%) than in previous reports. In a study in Greece, only 11.1% of ticks feeding on owned dogs were positive for endosymbionts (*Coxiella* or *Rickettsia*) or tick-borne pathogens (Latrofa et al., 2017). However, only adult ticks were processed in our study, being the stage in which more endosymbionts are detected (Latrofa et al., 2017), probably as a consequence of the multiple feeding of different stages, producing a higher bacterial load that is not specific of the tick, but "obtained" in the blood feeding of immatures. We acknowledge probable gaps in the design of this study, because every tick was collected while feeding on dogs. The presence of blood-derived endosymbionts DNA should be considered as a potential bias in the results obtained in this study.

Coxiella spp. endosymbionts have been found in *R. sanguineus* s.l., *I. hexagonus* and *I. ricinus*. All the pools of *R. sanguineus* s.l. and *I. hexagonus* yielded positive results for *Coxiella* spp., but a low prevalence was systematically recorded in *I. ricinus*, in line with previous publications (Estrada-Peña et al., 2015; Papa et al., 2017). Previous studies on the topic revealed a pronounced tropism of *Coxiella* spp. for the ovaries and the distal part of Malpighian tubules of the mentioned tick species (Bonnet et al., 2017; Duron et al., 2015; Guizzo et al., 2017). The ubiquity of *Coxiella* spp. in some genera of ticks, such as *Rhipicephalus*, could corroborate the hypothesis of an obligate endosymbiont (Duron et al., 2015, 2017; Guizzo et al., 2017; Papa et al., 2017). Some species could develop evolutionarily stable associations with their tick hosts (Bonnet et al., 2017; Duron et al., 2017; Estrada-Peña et al., 2016) resulting in coherent tick-endosymbiont phylogenies as observed between ticks belonging to the genus *Rhipicephalus* and their associated *Coxiella* endosymbionts (Bonnet et al., 2017; De la Fuente et al., 2017; Duron et al., 2017; Guizzo et al., 2017). These results should not be extrapolated to other species of ticks, since both previous studies and our results sharply demonstrated lower frequencies of *Coxiella* spp. in *I. ricinus* and *I. uriae* (Duron et al., 2015). In these tick species, *Coxiella* spp. could be more likely to behave as a conditional mutualist, but the confirmation of these roles warrants further research.

The second most prevalent endosymbiont detected in this study was *R. massiliae*, which was detected in 37% of the *R. sanguineus* s.l. pools. This rickettsial endosymbiont is included into the spotted fever group, which also includes *R. raoultii*, *Rickettsia aeschlimannii*, and *Rickettsia rhipicephali* (Chisu et al., 2018; Li et al., 2018; Moutailler et al., 2016; Papa et al., 2017; Zhong et al., 2007). Previous studies linked *R. massiliae* to cases of mild illnesses in dogs in California, and could cause spotted fever in human (Beeler et al., 2011; Chisu et al., 2018). Our data recorded *Rickettsia* endosymbionts in a large number of tick pools, supporting previous data in which most *Rickettsia* spp. were recorded exclusively in arthropods. *Rhipicephalus sanguineus* s.l. is one of the most frequent carriers (Bazzocchi et al., 2013; Duron et al., 2017; Estrada-Peña et al., 2016; Narasimhan and Fikrig, 2015; Perlman et al., 2006). This finding is consistent with previous reports where *Rhipicephalus* ticks were postulated to play an important role in the transmission of *Rickettsia* endosymbionts like *R. massiliae* (Chisu et al., 2018).

The high prevalence of *R. raoultii* among the *D. reticulatus* pools in the present study suggests that it could be an obligate endosymbiont. The presence of *R. raoultii* in *R. sanguineus* s.l. was detected for the first time in ticks collected from domestic dogs (Chisu et al., 2018), and this rickettsial organism could also play a low pathogenic role in some cases of spotted fever in vertebrates compared with *Rickettsia slovaca* (Parola et al., 2009). *Rickettsia raoultii* has mainly been found in *Dermacentor* ticks of European and Asiatic countries; however, as reported herein, other hard ticks such us *Rhipicephalus*, could be also carriers (Chisu et al., 2018; Špitalská et al., 2018).

Although the number of pools were low, our results corroborate that *R. monacensis*, a *Rickettsia* of the spotted fever group, could be associated with *I. ricinus* (Duron et al., 2015; Papa et al., 2017; Perlman et al., 2006). As previously reported, there is the possibility that this endosymbiont could also be acquired when the tick feeds on the mammalian host (Varela-Stokes et al., 2017). Therefore, the question about if it is a symbiotic organism intrinsically associated with the tick, and necessary of the metabolic functions of the arthropod, still remains.

Rickettsia sibirica mongolitimonae has been mainly reported in *Hyalomma* spp. ticks (Taylor et al., 2012; Varela-Stokes et al., 2017; Wang and Chandler, 2016). However, other data support the hypothesis that this *Rickettsia* circulates in *Rhipicephalus* spp. in the Iberian Peninsula (Ramos et al., 2013). The sequence found in the present study had 100% identity with sequences described in three human infections in the Mediterranean area of Spain (GenBank HQ710799 and HQ710800; Ramos et al., 2013). Our findings corroborate the contribution of the ticks of the genus *Rhipicephalus* as putative carriers of *R. sibirica mongolitimonae* displaying a widespread distribution in Spain.

As previously described by Li et al. (2018), our results confirm that *Coxiella* and *Rickettsia* were the predominant genera in feeding collected ticks. The explanation of this finding could be that these genera were transovarially transmitted, in contrast to other tick endosymbionts that are often obtained from the environment. Moreover, previous studies about *R. sanguineus* s.l. highlight the importance of the geographic origin, stages and tick genotypes in the endosymbiont composition. *Coxiella*-like endosymbionts are more prevalent in the western Africa, whereas the genus Ricketssia was found frequently in ticks from southern France (René-Martellet et al., 2017).

A sequence with 99% identity with '*Candidatus* Rickettsiella isopodorum', a new lineage of isopod-associated *Rickettsiella* bacteria previously reported by Kleespies et al. (2014) was recorded in 2 pools (out of 43) of *D. reticulatus*. We were unable to find further references regarding this lineage of *Rickettsiella* in ticks. Because the low number of specimens of the tick processed, we prefer to leave open the question of whether it refers to a casual association, or if this represents a new and yet unexplored association between a rickettsial organism and a tick.

The '*Candidatus* symbiont' described in *I. ricinus* was an endosymbiont widespread in this species of tick and similar to '*Candidatus* Midichloria mitochondrii' and '*Candidatus* Nicolleia massiliensis' (Ahantarig et al., 2013; Cafiso et al., 2016; Epis et al., 2008). '*Candidatus* Midichloria mitochondrii' has been described as a novel group of vector-borne agents, although pathogenicity for potential mammal hosts has not been demonstrated yet (Bazzocchi et al., 2013). The '*Candidatus* symbiont' found in *I. ricinus* appears to be ubiquitous in females and has a lower prevalence in males (Ahantarig et al., 2013), something that does not match our results. The discrepancy could be explained by the different relative abundance of *I. ricinus* sexes resulting in an obvious difference in the number of pools. This endosymbiont colonizes the cytoplasm of the ovarian cells and destroy organelles, specially the mitochondria, resulting in an infected offspring (Cafiso et al., 2016). The effect of this mechanism on the tick is yet unknown, however, it has been speculated that could produce some reproductive advantage (Cafiso et al., 2016; De la Fuente et al., 2017; Epis et al., 2008).

Our findings about *Francisella* as an alternative congenital tick endosymbiont for *D. reticulatus,* instead of *Coxiella* (6 pools out of 43) could be supported by previous studies (Ahantarig et al., 2013; Gall et al., 2016). In fact, *Francisella*-like endosymbionts have been identified in a wide range of species of *Dermacentor* (Ahantarig et al., 2013). Previous reports proposed a role for *Francisella* as a maternally

inherited tick endosymbiont similar to *Coxiella* (Duron et al., 2017), because almost all tick species without *Coxiella* infection have another maternally endosymbiont like *Francisella* (Duron et al., 2015; Estrada-Peña et al., 2015; Narasimhan and Fikrig, 2015). Evidence thus accumulates in line with our results.

As in previous publications (Latrofa et al., 2017; Varela-Stokes et al., 2017), ticks infected with classic tick-borne pathogens (*Babesia* spp., *Anaplasma* spp., *Theileria* spp., *C. felis, H. canis* and *Ehrlichia* spp.) constitute a low percentage of the total pools analyzed. Co-infections with multiple microorganisms (pathogens and symbionts) were detected in 129 out of 418 pools. The current study found a statistically significant association between the presence of *R. massiliae* in *R. sanguineus* s.l. and the absence of pathogens in the pools of the same tick, suggesting an "antagonist role". We acknowledge that the only way to establish congruent relationships among the detected bacteria, regarding possible events of transmission impairment, is to evaluate the physiological parameters of tick colonies with controlled endosymbiont faunal composition together with dedicated laboratory protocols. However, the presence of *R. massiliae* in *R. sanguineus* s.l could act similarly to rickettsial infections in *D. variabilis*, in which the inhibition of the transovarial transmission of a second *Rickettsia* has been reported (Ahantarig et al., 2013; De la Fuente et al., 2017; Li et al., 2018; Narasimhan and Fikrig, 2015; Zhong et al., 2007). Although *R. massiliae* is widespread in *R. sanguineus* s.l., it has also been recorded in *I. ricinus* (Bazzocchi et al., 2013; Bonnet et al., 2017; Epis et al., 2008).

5. Conclusions

This study provides a first overview of rickettsial and rickettsial-like endosymbiont fauna of ticks feeding on dogs in Spain. Our results suggest that few prevailing species such as *Coxiella* spp. and *Rickettsia* spp. largely dominated tick microbial communities, although specific microbiome profiles were not analyzed. This study confirms the presence of '*Candidatus* Rickettsiella isopodorum' in ticks in the western Mediterranean, and also reveals a *statistical* pattern supporting a negative relationship between *R. massiliae and* tick-borne pathogens. Furthermore, a DNA sequence compatible with *R. raoultii* was found in specimens of *D. reticulatus*. Additional studies should contribute to understand the physiological relationships of *Coxiella* and *Francisella* as maternally inherited endosymbionts. Further laboratory based on -omics technologies are needed to understand and characterize functional and evolutionary consequences of endosymbionts in ticks.

References

Ahantarig, A., Trinachartvanit, W., Baimai, V., Grubhoffer, L., 2013. Hard ticks and their bacterial endosymbionts (or would be pathogens). Folia Microbiol. 58, 419–428.

Amusátegui, I., Tesouro, M.A., Kakoma, I., Sainz, A., 2008. Serological reactivity to *Ehrlichia canis*, *Anaplasma phagocytophilum*, *Neorickettsia risticii*, *Borrelia burgdorferi* and *Rickettsia conorii* in dogs from northwestern Spain. Vector Borne Zoonotic Dis. 8, 797–803.

Bazzocchi, C., Mariconti, M., Sassera, D., 2013. Molecular and serological evidence for the circulation of the tick symbiont *Midichloria*(Rickettsiales: Midichloriaceae) in different mammalian species. Parasit. Vectors 6, 350.

Beeler, E., Abramowicz, K.F., Zambrano, M.L., Sturgeon, M.M., Khalaf, N., Hu, R., Dasch, G.A., Eremeeva, M.E., 2011. A focus of dogs and *Rickettsia massiliae*-infected *Rhipicephalus sanguineus* in California. Am. J. Trop. Med. Hyg. 84, 244–249.

Bonnet, S.I., Binetruy, F., Hernández-Jarguín, A.M., Duron, O., 2017. The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. Front. Cell. Infect. Microbiol. 7, 236.

Cafiso, A., Bazzochi, C., De Marco, L., Opara, M.N., Sassera, D., Plantard, O., 2016. Molecular screening for *Midichloria* in hard and soft ticks reveals variable prevalence levels and bacterial loads in different tick species. Ticks Tick Borne Dis. 7, 1186–1192.

Chisu, V., Foxi, C., Masala, G., 2018. First molecular detection of the human pathogen *Rickettsia raoultii* and other spotted fever group rickettsiae in Ixodid ticks from wild and domestic mammals. Parasitol. Res. 117, 3421–3429.

De la Fuente, J., Antunes, S., Bonnet, S., Cabezas-Cruz, A., Domingos, A.G., Estrada-Peña, A., Johnson, N., Kocan, K.M., Mansfield, K.L., Nijhof, A.M., Papa, A., Rudenko, N., Villar, M., Alberdi, P., Torina, A., Ayllón, N., Vancova, M., Golovchenko, M., Grubhoffer, L., Caracappa, S., Fooks, A.R., Gortazar, C., Rego, R.O.M., 2017. Tick-pathogen interactions and vector competence: identification of molecular drivers for tick-borne diseases. Front. Cell. Infect. Microbiol. 7, 114.

Duron, O., Noël, V., McCoy, K.D., Bonazzi, M., Sidi-Boumedine, K., Morel, O., Zenner, L., Jourdain, E., Durand, P., Arnathau, C., Renaud, F., Trape, J.F., Biguezoton, A.S., Cremaschi, J., Dietrich, M., Léger, E., Appelgren, A., Dupraz, M., Gómez- Díaz, E., Diatta, G., Dayo, G.K., Adakal, H., Zoungrana, S., Vial, L., Chevillo, C., 2015. The recent evolution of a maternally-inherited endosymbiont of ticks led to the emergence of the Q fever pathogen, Coxiella burnetii. PLoS Pathog. 11, 5.

Duron, O., Binetruy, F., Noël, V., 2017. Evolutionary changes in symbiont community structure in ticks. Mol. Ecol. 26, 2905–2921.

Epis, S., Sassera, D., Beninati, T., Lo, N., Beati, L., Piesman, J., Rinaldi, L., McCoy, K.D., Torina, A., Sacchi, L., Clementi, E., Genchi, M., Magnino, S., Bandi, C., 2008. *Midichloria mitochondrii* is widespread in hard ticks (Ixodidae) and resides in the mitochondria of phylogenetically diverse species. Parasitology 135, 485–494.

Estrada-Peña, A., de la Fuente, J., Ostfeld, R.S., Cabezas-Cruz, A., 2015. Interactions between tick and transmitted pathogens evolved to minimize competition through nested and coherent networks. Sci. Rep. 5, 10361.

Estrada-Peña, A., Sprong, H., Cabezas-Cruz, A., de la Fuente, J., Ramo, A., Coipan, E.C., 2016. Nested coevolutionary networks shape the ecological relationships of ticks, hosts, and the Lyme disease bacteria of the *Borrelia burgdorferi* (s.l.) complex. Parasit. Vectors 23, 517.

Estrada-Peña, A., Roura, X., Sainz, A., Guadalupe, M., Solano-Gallego, L., 2017. Species of ticks and carried pathogens in owned dogs in Spain: results of a one-year national survey. Ticks Tick Borne Dis. 8, 443–552.

Gall, C.A., Reif, K., Scoles, G.A., Mason, K.L., Mousel, M., Brayton, K.A., 2016. The bacterial microbiome of *Dermacentor andersoni* ticks influences pathogen susceptibility. ISME J. 10, 1846–1855.

Gray, J.S., Dautel, H., Estrada-Peña, A., Kahl, O., Lindgren, E., 2009. Effects of climate change on ticks and tick-borne diseases in Europe. Interdiscip. Perspect. Infect. Dis. 2009, 593232.

Guizzo, M.G., Parizi, L.F., Nunes, R.D., Schama, R., Albano, R.M., Tirloni, L., Oldiges, D.P., Vieira, R.P., Oliveira, W.H.C., Leite, M.S., Gonzales, S.A., Farber, M., Martins, O., Vaz, I.D.S., Oliveira, P.L., 2017. A *Coxiella* mutualist symbiont is essential to the development of *Rhipicephalus microplus*. Sci. Rep. 7, 17554.

Kleespies, R.G., Federici, B.A., Leclerque, A., 2014. Ultrastructural characterization and multilocus sequence analysis (MLSA) of '*Candidatus Rickettsiella isopodorum*', a new lineage of intracellular bacteria infecting woodlice (Crustacea: Isopoda). Syst. Appl. Microbiol. 37, 351–359.

Kurlovs, A.H., Li, J., Cheng, D., Zhong, J., 2014. *Ixodes pacificus* ticks maintain embryogenesis and egg hatching after antibiotic treatment of *Rickettsia* endosymbiont. PLoS One 9, e104815.

Latrofa, M.S., Angelou, A., Giannelli, A., Annoscia, G., Ravagnan, S., Dantas-Torres, F., Capelli, G., Halos, L., Beugnet, F., Papadoulos, E., Oranto, D., 2017. Ticks and associated pathogens in dogs from Greece. Parasit. Vectors 10, 301.

Li, L., Zhang, Y., Zhu, D., 2018. Effects of antibiotic treatment on the fecundity of *Rhipicephalus haemaphysaloides* ticks. Parasit. Vectors 11, 242.

Michelet, L., Joncour, G., Devillers, E., Torina, A., Vayssier-Taussat, M., Bonnet, S.I., Moutailler, S., 2016. Tick species, tick-borne pathogens and symbionts in an insular environment off the coast of Western France. Ticks Tick Borne Dis. 7, 1109–1115.

Miró, G., Montoya, A., Roura, X., Gálvez, R., Sainz, A., 2013. Seropositivity rates for agents of canine vector-borne diseases in Spain: a multicentre study. Parasit. Vectors 6, 117.

Moutailler, S., Valiente, M., Vaumourin, E., Michelet, L., Tran, F.H., Devillers, E., Cosson, J.F., Gasqui, P., Van, V.T., Mavingui, P., Vourc'h, G., Vayssier-Taussat, M., 2016. Co-infection of ticks: the rule rather than the exception. PLoS Negl. Trop. Dis. 10, e0004539.

Narasimhan, S., Fikrig, E., 2015. Tick microbiome: the force within. Trends Parasitol. 31, 315–323.

Nava, S., Beati, L., Venzal, J.M., Labruna, M.B., Szabó, M.P.J., Petney, T., Saracho-Bottero, M.N., Tarragona, E.L., Dantas-Torres, F., Silva, M.M.S., Mangold, A.J., Guglielmone, A.A., Estrada-Peña, A., 2018. *Rhipicephalus sanguineus* (Latreille, 1806): neotype designation, morphological re-description of all parasitic stages and molecular characterization. Ticks Tick Borne Dis. 9, 1573–1585.

Papa, A., Tsioka, K., Kontana, A., Papadopoulos, C., Giadinis, N., 2017. Bacterial pathogens and endosymbionts in ticks. Ticks Tick Borne Dis. 8, 31–35.

Parola, P., Rovery, C., Rolain, J.M., Brouqui, P., Davoust, B., Raoult, D., 2009. *Rickettsia slovaca* and *R. raoultii* in tick-borne rickettsioses. Emerg. Infect. Dis. 15, 1105–1108.

Perlman, S.J., Hunter, M., Zchori-Fein, E., 2006. The emerging diversity of Rickettsia. Proc. Biol. Sci. 273, 2097–2106.

Ramos, R.A., Giannelli, A., Brianti, E., Annoscia, G., Cantacessi, C., Dantas-Torres, F., Oranto, D., 2013. Tick vectors of *Cercopithifilaria bainae* in dogs: *Rhipicephalus sanguineus* sensu lato versus Ixodes ricinus. Parasitol. Res. 112, 3013–3017.

René-Martellet, M., Minard, G., Massot, R., Van, V.T., Valiente-Moro, C., Chabanne, L., Mavingui, P., 2017. Bacterial microbiota associated with *Rhipicephalus sanguineus* (s.l.) ticks from France, Senegal and Arizona. Parasit. Vectors 10, 416.

Špitalská, E., Sparagano, O., Stanko, M., Schwarzová, K., Špitalsky, Z., Škultéty, L., Havlíková, Sf., 2018. Diversity of *Coxiella*-like and *Francisella*-like endosymbionts, and *Rickettsia* spp., *Coxiella burnetii* as pathogens in the tick populations of Slovakia, Central Europe. Ticks Tick Borne Dis. 9, 1207–1211.

Taylor, M., Mediannikov, O., Raoult, D., Greub, G., 2012. Endosymbiotic bacteria associated with nematodes, ticks and amoebae. FEMS Immunol. Med. Microbiol. 64, 21–31.

Varela-Stokes, A.S., Park, S.H., Kim, S.A., Ricke, S.C., 2017. Microbial communities in North American Ixodid ticks of veterinary and medical importance. Front. Vet. Sci. 4, 179.

Wang, Y.D., Chandler, C., 2016. Candidate pathogenicity islands in the genome of '*Candidatus* Rickettsiella isopodorum', an intracellular bacterium infecting terrestrial isopod crustaceans. Peer J. 4, e2806.

Zhong, J., Jasinskas, A., Barbour, A.G., 2007. Antibiotic treatment of the tick vector *Amblyomma americanum* reduced reproductive fitness. PLoS One 2, e405.