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# Characterization of the bacterial microbiota in wild-caught *Ixodes ventalloi*

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ABSTRACT

Exploring the microbial diversity of ticks is crucial to understand geographical dispersion and pathogen transmission. Tick microbes participate in many biological processes implicated in the acquisition, maintenance, and transmission of pathogens, and actively promote host phenotypic changes, and adaptation to new environments. The microbial community of *Ixodes ventalloi* still remains unexplored. In this study, the bacterial microbiota of wild-caught *I. ventalloi* was characterized using shotgun-metagenomic sequencing in samples from unfed adults collected during December 2013-January 2014 in two locations from Sicily, Italy. The microbiota identified in *I. ventalloi* was mainly composed of symbiotic, commensal, and environmental bacteria. Interestingly, we identified the genera *Anaplasma* and *Borrelia* as members of the microbiota of *I. ventalloi*. These results advance our information on *I. ventalloi* microbiota composition, with potential implications in tick-host adaptation, geographic expansion, and vector competence.

## 1. Introduction

Ticks and tick-borne diseases represent a major health issue (Jongejan and Uilenberg, 2004). Scientists around the world are concerned about how the increase of human activities, fragmentation of wildlife habitat and global warming have incremented the opportunities for ticks to expand, colonizing new territories, and increase the contacts with new hosts (Allen et al., 2003; Dantas-Torres, 2015; Estrada-Peña et al., 2014; Narasimham and Fikring, 2015). Microbes form part of the tick hologenome, and thus participate in many biological processes directly linked to vector eco-epidemiological dynamics (Ahantarig et al., 2013; Clay and Fuqua, 2010; Narasimhan et al., 2014; Bonnet et al., 2017). One of the key aspects for researchers is to decipher how the tick microbial community assists and modulates the acquisition, maintenance, and transmission of pathogens (Carpi et al., 2011; de la Fuente et al., 2017; Abraham et al., 2017). Recent trends in metagenomics have permitted to explore the implication of tick-associated microbiota in the development of adaptive skills. One of the keys is the rapid adaptation of microbes to new environments, accelerating host phenotypic changes, which in turn facilitates adaptation (Brucker and Bordenstein, 2013; de la Fuente et al., 2016; Sevellec et al., 2018). Traditionally, tick-endosymbionts and their cooperative relationship have received most of the researchers´ attention (Cowdry, 1925; Ahantarig et al., 2013; de la Fuente et al., 2016; Duron et al., 2017). Nevertheless, the diversity of the tick-associated microbiota is high, and microbes acquired from the environment likely have an impact in diverse metabolic networks, stimulate microbial alliances, and host functions (Zolnik et al., 2016; de la Fuente et al., 2016, 2017). In recent years, the interest on “who” of the tick microbes and “how” they are implicated in vector competence (i.e. a component of vectorial capacity that depends on genetic factors affecting the ability of a vector to transmit a pathogen) is growing to better design tick-borne diseases management strategies (Greay et al., 2018).

So far, the tick-associated microbiota of the genus *Ixodes* (Linnaeus, 1758) has only been described in the species *I. scapularis*, *I. ricinus, I. persulcatus, I. pavlovskyi* and *I. ovatus* by 16S amplicon sequencing studies (Qiu et al., 2014; Eshoo et al., 2015; Kurilshikov et al., 2015; Rynkiewicz et al., 2015; van Treuren et al., 2015; Sui et al., 2017; Clow et al., 2018; Greay et al., 2018; Hernández-Jarguín et al., 2018). However, the tick-associated microbiota of many *Ixodes* species has not been described yet.

The rabbit tick, *I. ventalloi* (Gil Collado, 1936) is one of those *Ixodes*

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species whose associated microbiota is still unexplored. From decades, medical relevance of *I. ventalloi* has been questioned as originally this tick was restricted to parasitize the European wild rabbit (*Oryctolagus cuniculus*). However, recent reports have warned about the potential vector competence of *I. ventalloi* to transmit pathogens (Santos et al., 2004; Latrofa et al., 2017; Huber et al., 2017), adapt to new hosts (Santos Dias and Santos-Reis, 1989; Otranto et al., 2014; Pennisi et al., 2015; Latrofa et al., 2017), and geographic expansion (Chastel et al., 1984; Petney et al., 1996; Jameson and Medlock, 2011; Santos-Silva et al., 2006 2011). Novel whole-genome shotgun metagenomics can be used to characterize the microbiota composition of tick vectors by sequencing the genome of all microorganisms present in the sample (Carpi et al., 2011).

Herein, we present the first characterization of the bacterial microbiota of adult wild-caught *I. ventalloi*. Specifically, we used a methodology based on Illumina-technology as an attempt to broad screen the bacterial microbiota composition and the load of common tick-borne pathogens of *I. ventalloi*. Finally, the tick bacterial microbial-rich lineages that compose the putative microbiota of *I. ventalloi* will provide insights into potential host-microbiome interactions participating in vector competence.

## 2. Material and methods

### 2.1. Sample collection and study model

Unfed host-seeking adult female *I. ventalloi* ticks were collected during December 2013 and January 2014 from two localities in Sicily (Italy), within the Natural Area of Monte Pellegrino. Monte Pellegrino is a natural reserve that extends over an area of 1300 ha bordering Palermo and extending to the Mediterranean Sea, characterized by a Mediterranean climate and a diverse flora and fauna (Raimondo and Venturella, 1993a,b;Surano et al., 1993). The two localities selected for tick collection, Boschetto Airoldi (Location 1; Long 13.35141°, Lat 38.14946°, 35 m a.s.l.) and Castello Utveggio (Location 2; Lon 13.35469,º Lat 38.15640°, 280 m a.s.l.), can be described as an artificial forest of pine and eucalyptus and the ubiquitous presence of rabbits (*Oryctolagus cuniculus*, Linnaeus, 1758), rodents (*Rattus norvegicus*, Berkenhout, 1769) and some species of Canidae (Torina et al., 2018).

One thousand and twenty-five total ticks were collected from these two locations. Collection of host-seeking ticks was conducted from vegetation using a 1 $m^2$ flannel drag cloth that was dragged along the floor and surrounding vegetation. Ticks were identified to the genus and species levels by using a steromicroscope, according to the standard keys given by Manilla (1998). A total of 125 ticks were finally washed in 70% ethanol and sterile distilled water to reduce background contamination. Whole ticks were then cut and crushed with a sterile scalpel, combined with lysis buffer and shaken on a vortex with glass beads. Pooling individual *I. ventalloi* ticks was required to obtain good DNA extraction yields. Finally, ticks were grouped according to location for subsequent analysis. In this project, we aim to identify unique and common taxa in a particular tick species, so two biological replicates were taken on each location for consistently obtain biological information (Fig. 1).

### 2.2. Performance of whole-genome shotgun metagenomics sequencing

Genomic DNA was extracted from two biological replicates of pooled samples with a total of 100 tick specimens for Location 1, and 25 tick specimens for Location 2. Finally two biological replicates with 50 pooled specimens each for Location 1, and 12 and 13 pooled specimens each for Location 2 were used for further analysis (Fig. 1). Whole ticks were ground and pulverized in liquid nitrogen and homogenized using a glass homogenizer (20 strokes) in 4 ml buffer (0.25 sucrose, 1 mM $MgCl_2$, 10 mM Tris – HCl, pH 7.4), supplemented with 4% SDS and complete mini protease inhibitor cocktail (Roche, Basel, Switzerland). DNA was extracted using the NucleoSpin TriPrep kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions and quantified using PicoGreen (Invitrogen, Carlsbad, CA, USA) for template ranges of 4 ng to 100 ng. An aliquot of each pooled replicate was adjusted to the same DNA concentration and exhaustively fragmented using a BioRuptor (Diagenode, Inc, NJ, USA). Libraries were prepared using the Ultra DNA library preparation kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer´s instructions. Pooled samples were indexed using a unique combination of primers provided by the manufacturer. After library preparation, DNA library was purified in 1.5% agarose gels to select fragments with sizes within 150–400 bp, quantified with the Bioanalyzer TM 2100 (Agilent Technologies, Palo Alto, CA, USA) and then pooled in equimolar concentrations. In addition, to prepare for cluster generation and sequencing, equal volumes of normalized libraries were combined, diluted in hybridization buffer and heat denatured, according to Illumina protocol (Illumina, Inc, San Diego, CA, USA). Finally, pair-end sequencing was performed on the HiSeq2000 platform (Illumina, San Diego, CA, USA) using the TruSeq reagent kit (2 × 100 bp) according to the manufacturer´s instructions (Fig. 1).

### 2.3. Sequencing data analysis

For assembly of the metagenomics reads, raw data files from shotgun sequencing were de-multiplexed, filtered by quality and converted to fastq using Casava v.1.8.2 (Illumina). High-quality reads were processed using a metagenomic *de novo* assembly approach. First, a filtering analysis was performed in order to remove the arthropod reads.

Raw reads from *I. ventalloi* were mapped using Bowtie2 software (http://bowtie-bio.sourceforge.net/index.shtml) (Langmead et al., 2009) against *Ixodes scapularis* complete genome assembled (VectorBase, https://www.vectorbase.org). All the unmapped reads remaining after vector filtering (14 to 22 million reads per sample representing 24–28% of the total; Supplementary file 1: TS2) were assembled using SPADES with a minimum contig length of 200 bp (http://bioinf.spbau.ru/spades). The quality of genome assemblies corresponding to the *I. ventalloi* metagenome was further evaluated using QUAST (http://bioinf.spbau.ru/quast)" (Supplementary file 1: FS1). And finally were searched and annotated against a microbial database previously constructed with bacterial sequences of species-specific ribosomal RNA (rRNA) sequences downloaded from the NCBI (https://www.ncbi.nlm.nih.gov) (Supplementary file 1: TS1). The bioinformatics approach to identify bacterial sequences was done in two steps. First, we used LAST genome-scale sequence comparison tool (http://last.cbrc.jp) to search against the bacterial database previously constructed. As cut off criteria for LAST we applied minimum alignment length of 100 nt, e-value 0.001, with a word size of 11, and a minimum of 70% sequence identity. Then, putative bacterial reads detected with LAST were further confirmed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE = BlastSearch) (Frith et al., 2010a, b; Kiełbasa et al., 2011). BLAST assignments were done by using the 10 best BLAST hits (BBH) for each putative bacteria previously assigned by LAST. The sequences with hits matching to bacteria were confirmed as identified bacterial sequences, discarding the rest (Fig. 1).

The microbiota composition was reported in terms of relative abundance of the taxa identified, and defined by the proportion of reads mapping to the microbial bacteria database previously constructed (Supplementary file 1: TS1). Taxonomic classification was based on multigene assignments, 16S rRNA and intergenic regions. The taxonomic relative abundance was calculated as the total number of reads mapping to each bacteria genome, and normalized against the total number of reads. The taxonomic relative abundance was assessed separately for each biological replicate and for each location. Finally, bacterial taxonomic assignments at phyla and genus level were ranked based on the ratio of the average relative abundance across biological

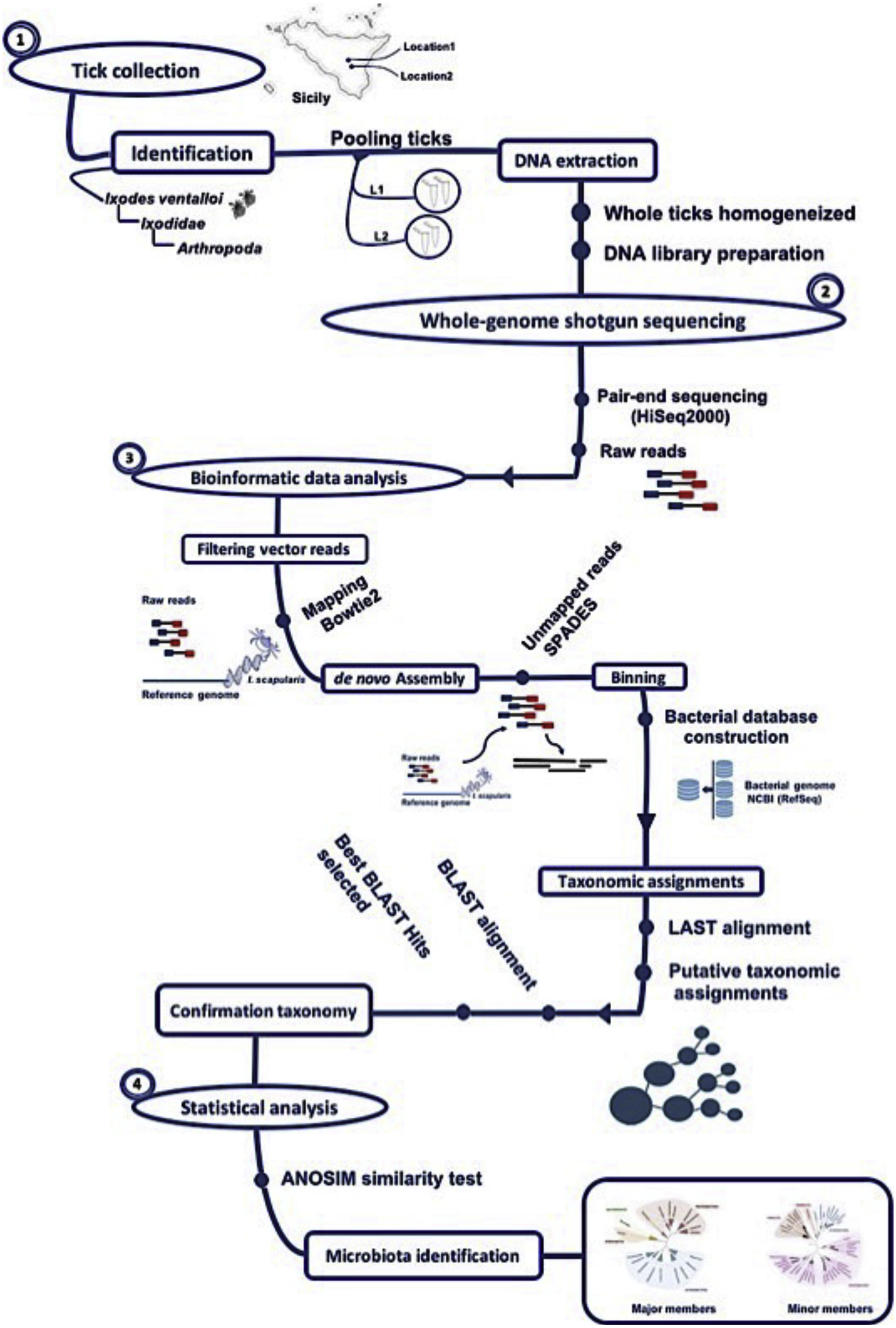

**Fig. 1.** Workflow scheme for *I. ventalloi* microbiota identification.

replicates for each location (Fig. 1; Supplementary file 2: Dataset S1). Further classification of bacterial taxa at genus level was done as follows: genera were classified as major members of the microbiota when the ratio of taxonomic relative abundance was ≥ 1.00%. As well, genera were classified as minor members of the microbiota when the ratio of taxonomic relative abundance was between 0.10 and 1.00%. The bacterial genera that represented less than a 0.10% in the microbiota can be found as well, in Table 1.

To test differential abundance of the taxonomic assignments within each location, the biological replicates were compared on the basis of univariate statistical comparisons by a Mann-Whitney/Kruskal-Wallis analysis (adjusted p-value cut-off 0.05). Finally, the putative microbiota to both locations were compared by ANOSIM analysis and Bray-Curtis dissimilarity that were performed at genus level using the software R v.1.1.38, and the anosim function within the vegan package. Tick-associated microbiota composition was visually assayed with pruned cladograms displayed using the open source platforms PhyloT based on the NCBI taxonomy names (http://phylot.biobyte.de) and iTOL (https://itol.embl.de) open source platforms.

## 3. Results and discussion

All the data obtained from the shotgun metagenomic sequencing of whole tick bodies contributed to structure the microbiota composition of *I. ventalloi* including pathogenic symbiotic and non-pathogenic bacteria.

The bacterial microbiota analysis of the pooled samples represented a total of 125 *I. ventalloi* ticks, which were further processed and sequenced. After the run, sequences were demultiplexed and filtered by quality, which finally rendered about 250 millions pair-end reads per sample. Overall, 73,976,098 million pair-end reads remained after vector filtering, cleaning and quality processing. Unidentified reads were not included in the analysis, and 1–6704 reads (average ± SE, 124 ± 32 Location 1, 229 ± 71 Location 2) were used for genera classification (Supplementary file 2: Dataset S1). Finally, the metagenomics sequencing revealed that the bacteria taxonomic composition of all dataset included a total of 6 phyla, 71 families and 107 genera (Supplementary file 2: Dataset S1). The phyla Actinobacteria (48.95%) and Proteobacteria (44.83%) were predominant over the phyla Bacteroidetes (2.59%), Firmicutes (1.92%) and Spirochaetes (1.64%), meanwhile the phyla Tenericutes (0.41%) was scarcely represented.

The differential abundance analysis showed no statistical differences between the biological replicates composing each location (Mann-Whitney/Kruskal-Wallis, p > 0.05). This result was supported by the ANOSIM and Bray-Curtis dissimilarities analysis performed at the genus level (R= -0; p = 0.66) which revealed a similar microbiota composition, leading us to speculate with the existence of a putative microbiota present in wild-caught *I. ventalloi.* Whether or not geographic location and/or environment are important drivers of differences in the microbiota of *I. ventalloi* needs to be further addressed. Within the putative microbiota observed, the major members of the microbiota included 17 families and 19 genera (Table 1; Fig. 2A). And a total of 31 families and 43 genera composed the so-called minor members of the microbiota (Table 1; Fig. 2B).

Among all microbiota members of *I. ventalloi*, the bacteria genera *Anaplasma* and *Borrelia* were detected within the major microbiota (Table 1; Fig. 2A). These dominant genera includes from species that are the causative agents of diseases such as human granulocytic anaplasmosis (*A. phagocytophilum*), Lyme borreliosis (*B. burgdorferi* s.l.), and tick-borne relapsing fever (e.g. *B. miyamotoi*) (Estrada-Peña et al., 2012; Breuner et al., 2017), to non-pathogenic species and species of undetermined pathogenicity (Anderson et al., 1990; Portillo et al., 2005; Margos et al., 2011;2014). *I. ventalloi* is not the classic vector of *Anaplasma* and *Borrelia*, despite previous studies suggest that *I. ventalloi* might be a suitable host and somehow takes part in their eco-epidemiological cycles, or even cooperate for a mutual benefit (Santos et al.,

**Table 1**
Bacterial microbiota composition of *I. ventalloi.* Genus level composition is reported in terms of average percentage of relative abundance.

| MAJOR MICROBIOTA[*] | | | |
|---|---|---|---|
| Phylum | Family | Genus | Relative abundance (%) |
| Actinobacteria | *Corynebacterineae* | *Rhodococcus* | 9.89 |
| | *Geodermatophilaceae* | *Blastococcus* | 1.29 |
| | *Mycobacteriaceae* | *Mycobacterium* | 10.68 |
| | *Nocardioidaceae* | *Aeromicrobium* | 2.60 |
| | *Propionibacteriaceae* | *Cutibacterium* | 14.76 |
| | *Pseudonocardiaceae* | *Amycolatopsis* | 2.02 |
| | | *Actinosynema* | 1.00 |
| | | *Pseudonocardia* | 1.21 |
| | *Tsukamurellacea* | *Tsukamurella* | 2.07 |
| Bacteroidetes | *Bacteroidaceae* | *Bacteroides* | 2.52 |
| Proteobacteria | *Anaplasmataceae* | *Anaplasma* | 7.48 |
| | *Caulobacteraceae* | *Brevundimonas* | 2.15 |
| | *Comamonadaceae* | *Variovorax* | 2.10 |
| | *Enterobacteriaceae* | *Escherichia* | 7.75 |
| | *Moraxellaceae* | *Moraxella* | 2.67 |
| | *Pseudomonadaceae* | *Pseudomonas* | 2.82 |
| | *Rickettsiaceae* | *Rickettsia* | 9.29 |
| | *Sphingomonadaceae* | *Sphingomonas* | 2.13 |
| Spirochaetes | *Spirochaetaceae* | *Borrelia* | 1.64 |
| MINOR MICROBIOTA[**] | | | |
| Phylum | Family | Genus | Relative abundance (%) |
| Actinobacteria | *Conexibacteraceae* | *Conexibacter* | 0.36 |
| | *Dermabacteraceae* | *Brachybacterium* | 0.29 |
| | *Dietziaceae* | *Dietzia* | 0.46 |
| | *Microbacteriaceae* | *Clavibacter* | 0.07 |
| | *Micrococcaceae* | *Arthrobacter* | 0.21 |
| | | *Rothia* | 0.26 |
| | *Micromonosporaceae* | *Actinoplanes* | 0.97 |
| | | *Micromonospora* | 0.43 |
| | *Propionibacteriaceae* | *Propionibacterium* | 0.23 |
| Firmicutes | *Bacillaceae* | *Bacillus* | 0.21 |
| | *Erysipelotrichaceae* | *Erysipelothrix* | 0.10 |
| | *Lachnospiraceae* | *Roseburia* | 0.14 |
| | *Peptoniphilaceae* | *Anaerococcus* | 0.06 |
| | | *Finegoldia* | 0.12 |
| | *Streptococcaceae* | *Streptococcus* | 0.53 |
| | *Veillonellaceae* | *Veillonella* | 0.10 |
| Tenericutes | *Spiroplasmataceae* | *Spiroplasma* | 0.41 |
| Proteobacteria | *Acetobacteraceae* | *Roseomonas* | 0.30 |
| | *Aeromonadaceae* | *Aeromonas* | 0.14 |
| | *Alcaligenaceae* | *Achromobacter* | 0.39 |
| | | *Bordetella* | 0.10 |
| | *Bradyrhizobiaceae* | *Afipia* | 0.18 |
| | | *Bradyrhizobium* | 0.44 |
| | | *Rhodopseudomonas* | 0.24 |
| | *Burkholderiaceae* | *Burkholderia* | 0.19 |
| | | *Ralstonia* | 0.16 |
| | *C. Midichloriaceae* | *C. Midichloria* | 0.22 |
| | *Caulobacteraceae* | *Caulobacter* | 0.80 |
| | *Comamonadaceae* | *Acidovorax* | 0.85 |
| | *Enterobacteriaceae* | *Enterobacter* | 0.17 |
| | | *Klebsiella* | 0.24 |
| | | *Salmonella* | 0.11 |
| | *Moraxellaceae* | *Acinetobacter* | 0.66 |
| | *Pasteurellaceae* | *Mannheimia* | 0.23 |
| | *Phyllobacteriaceae* | *Mesorhizobium* | 0.31 |
| | *Rhizobiaceae* | *Agrobacterium* | 0.25 |
| | | *Rhizobium* | 0.23 |
| | | *Sinorhizobium* | 0.31 |
| | *Rhodobacteraceae* | *Rhodobacter* | 0.14 |
| | *Rhodocyclaceae* | *Thauera* | 0.11 |
| | | *Novosphingobium* | 0.43 |
| | *Xanthomonadaceae* | *Pseudoxanthomonas* | 0.49 |
| | | *Stenotrophomonas* | 0.30 |
| LESS REPRESENTED BACTERIA[***] | | | |
| Phylum | Family | Genus | Relative abundance (%) |

(*continued on next page*)

**Table 1** (*continued*)

| MAJOR MICROBIOTA* | | | |
|---|---|---|---|
| Actinobacteria | *Actinomycetaceae* | *Actinomyces* | 0.02 |
| | *Bifidobacteriaceae* | *Bifidobacterium* | 0.05 |
| | *Micrococcaceae* | *Haematomicrobium* | 0.05 |
| Bacteroidetes | *Flavobacteriaceae* | *Flavobacterium* | 0.03 |
| | *Chitinophagaceae* | *Niastella* | 0.01 |
| | *Sphingobacteriaceae* | *Pedobacter* | 0.02 |
| | *Prevotellaceae* | *Prevotella* | 0.01 |
| | *Cytophagaceae* | *Runella* | 0.01 |
| Deinococcus-Thermus | *Deinococcaceae* | *Deinococcus* | 0.03 |
| Firmicutes | *Bacillaceae* | *Anoxybacillus* | 0.04 |
| | *Clostridiaceae* | *Clostridium* | 0.01 |
| | *Lachnospiraceae* | *Dorea* | 0.01 |
| | *Enterococcaceae* | *Enterococcus* | 0.06 |
| | *Listeriaceae* | *Listeria* | 0.01 |
| | *Veillonellaceae* | *Megasphaera* | 0.02 |
| | *Staphylococcaceae* | *Staphylococcus* | 0.09 |
| | *Leuconostocaceae* | *Weissella* | 0.01 |
| | *Ruminococcaceae* | *Faecalibacterium* | 0.02 |
| Fusobacteria | *Fusobacteriaceae* | *Fusobacterium* | 0.01 |
| Proteobacteria | *Campylobacteraceae* | *Arcobacter* | 0.02 |
| | *Acetobacteraceae* | *Asaia* | 0.01 |
| | *Caulobacteraceae* | *Asticcacaulis* | 0.04 |
| | *Bartonellaceae* | *Bartonella* | 0.01 |
| | *Brucellaceae* | *Brucella* | 0.01 |
| | *Pseudomonadaceae* | *Cellvibrio* | 0.001 |
| | *Enterobacteriaceae* | *Citrobacter* | 0.02 |
| | *Coxiellaceae* | *Coxiella* | 0.01 |
| | *Anaplasmataceae* | *Ehrlichia* | 0.04 |
| | *Erwiniaceae* | *Erwinia* | 0.04 |
| | *Francisellaceae* | *Francisella* | 0.002 |
| | *Acetobacteraceae* | *Gluconobacter* | 0.01 |
| | *Legionellaceae* | *Legionellaceae* | 0.01 |
| | *Myxococcaceae* | *Myxococcus* | 0.04 |
| | *Pasteurellaceae* | *Pasteurella* | 0.003 |
| | *Morganellaceae* | *Proteus* | 0.004 |
| | | *Providencia* | 0.01 |
| | *Yersiniaceae* | *Serratia* | 0.07 |
| | *Enterobacteriaceae* | *Shimwella* | 0.001 |
| | *Polyangiaceae* | *Sorangium* | 0.07 |
| | *Vibrionaceae* | *Vibrio* | 0.003 |
| | *Rickettsiaceae* | *Wolbachia* | 0.004 |
| | *Xanthomonadales* | *Xylella* | 0.01 |
| | *Yersiniaceae* | *Yersinia* | 0.01 |
| | *Sphingomonadaceae* | *Zymomonas* | 0.01 |
| Spirochaetes | *Spirochaetaceae* | *Treponema* | 0.05 |

* **Major Microbiota** composition of *I. ventalloi.* Genus level composition is reported in terms of average relative abundance with values of ≥ 1.00%.

** **Minor Microbiota** composition of *I. ventalloi*. Genus level composition is reported in terms of average relative abundance with values of 0.10%- 1.00%.

*** **Less Represented Microbiota** found in *I. ventalloi*. Genus level composition is reported in terms of relative abundance with values of less than 0.10%.

2004; Tomassone et al., 2013; Bonnet et al., 2017). However, it should be noted that our analysis is not a reflection of the bacterial microbiota at species level. The major microbiota contained the genus *Rickettsia* as well (Table 1; Fig. 2A). This genus is a common member of the *Ixodes* midgut microbiota (Nakao et al., 2013; Vayssier-Taussat et al., 2013; Naramsiham et al., 2014; Qiu et al., 2014; Hernández-Jarguín et al., 2018), and as a result of co-evolution it can show a dual behavior as pathogen or endosymbiont (Carpi et al., 2011; Hunter et al., 2015; Bonnet et al., 2017). It is evident that the presence of this bacterium within the microbiota might influence vector competence. Whether or not *I. ventalloi* is capacitated to maintain and transmit these pathogens it is a matter of debate.

Other cohabiting members of the major microbiota detected, were the ubiquitous genera, *Sphingomonas* and *Pseudomonas* (Table 1; Fig. 2A). These genera have been previously catalogued with the status "symbiont", due to the high rates of prevalence frequently observed in ticks (Qiu et al., 2014; Narasimhan et al., 2015; Van Treuren et al., 2015; Sui et al., 2017; Clow et al., 2018). Members of the genus *Pseudomonas* have been linked to the maintenance of the midgut redox homeostasis in blood-feeding insects (Alvarez et al., 2012). Following these abundant symbionts, we detected much lower abundance of the genera *Acinetobacter* and *Spiroplasma*, (Table 1; Fig. 2B). Both genera are frequently reported in *Ixodes* i.e., *I. ovatus, I. persulcatus*, *I. ricinus* and I. *pavlovskyi* (Tully et al., 1981; Taylor et al., 2015; Qiu et al., 2014; Bell-Sakyi et al., 2015; Kurilshikov et al., 2015), as facultative symbiotic bacteria (Lysyk et al., 1999; Qiu et al., 2014; Moutailler et al., 2016). However their lifestyle is controversial and switches from commensals and symbiotic lifestyle to become in pathogenic to human and animals (Henning et al., 2006; Lo et al., 2015).

Interestingly, we also observed the obligate intracellular tick endosymbiont *Candidatus* Midichloria mitochondrii but represented by very low rates within the minor members of the microbiota (Table 1; Fig. 2B). This intracellular symbiont is well-described in *I. ricinus* (Sassera et al., 2006). However, in other hard ticks the presence of this symbiotic bacterium is very variable or absent (Epis et al., 2008; Beninati et al., 2009). In field-collected *I. ricinus* females a 100% prevalence of *C.* Midichloria mitochondrii was found in ovarian tissues, however, in males the prevalence decrease notably across maturation (Lo et al., 2006; Njam et al., 2012). Therefore, the low presence of *C.* Midichloria mitochondrii observed in our study might be explained by the effect of pooling male and female ticks, which likely reduced the chances to identify this bacterium. Nevertheless, the association of this bacterium with *I. ventalloi* should be further explored before conjecture any symbiotic interaction.

Other genera composing the minor microbiota included some pathogenic species for animals and humans, including *Bordetella*, *Klebsiella*, *Salmonella*, *Erysiphelotrix*, *Mannheimia*, and *Streptococcus* (Table 1; Fig. 2B). Most of these genera are common members of adult tick´s midgut microbiota, and their presence is coherent with co-infections likely acquired from the environment and/or from their on-host feeding habits (Carpi et al., 2011; Otranto et al., 2014; Pennisi et al., 2015). But at the same time, tick´s co-infection can be linked to co-transmission of these pathogens to both human and animals, which might result in disease severity and/or interfere in diagnosis (Moutailler et al., 2016).

We detected the dominant genus *Cutibacterium* (formerly *Propionibacterium*) at high rates within the major microbiota (Table 1; Fig. 2B). Colonization of ticks by skin bacteria from humans is a common finding in the microbiota of blood-feeding arthropods. In particular, *Cutibacterium* resides in the skin sebaceous glands skin releasing volatile molecules that result attractive to blood-feeding arthropods, that might conditionate host-seeking behavior (Niels et al., 2018).

Besides pathogenic bacteria, naturally-occurring bacteria were detected within the minor microbiota, including the genera *Bacillus*, *Bradyrhizobium, Novosphingobium, Agrobacterium,* and *Stenotrophomonas* (Table 1; Fig. 2B). All these genera are frequent members of the soil and water microbial communities that colonize tick´s breeding sites, but also these bacteria are described from contamination of DNA extraction kits (Narasimhan et al., 2014; Salter et al., 2014; Carpi et al., 2011). However, the notion of this bacterium as true microbial partners and its colonization pathways is not well understood, as they can be selectively acquired commensals or occasional colonizers of tick´s midguts.

## 4. Conclusion

In this study we characterized the composition of bacterial microbiota in *I. ventalloi* field collected from two locations in Sicily, Italy. Additionally, the putative microbiota of *I. ventalloi* was described using shotgun metagenomic methodology detecting pathogenic, symbiotic and non-pathogenic agents. The first identification of the tick bacterial microbiota is a fundamental step for further explore microbial

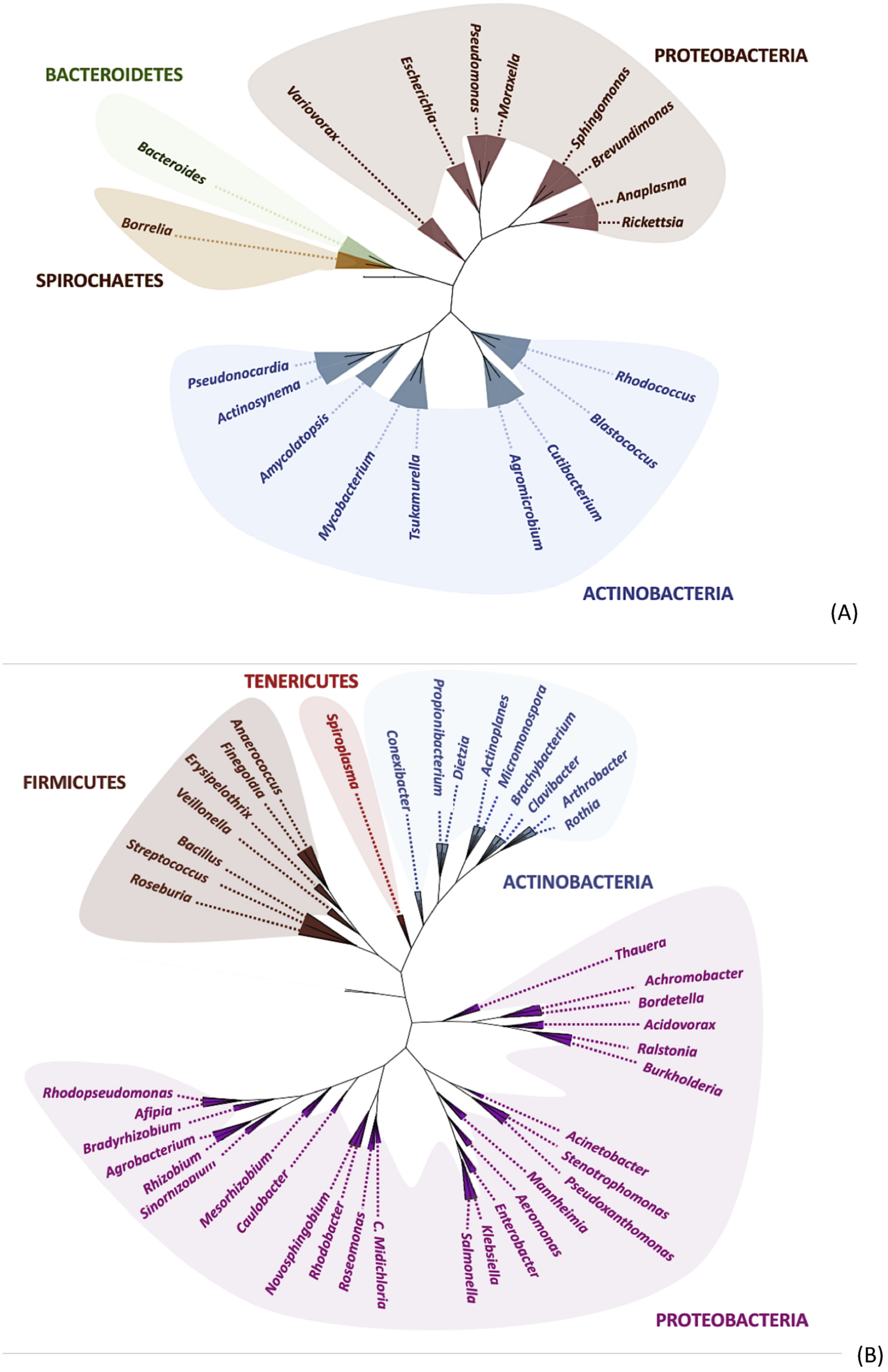

**Fig. 2.** Unrooted phylogenetic tree showing the major (A) and the minor (B) microbiota identified in *I. ventalloi*. The phylogenetic tree was generated with PhyloT and visualized with iTOL.

functionality and interactions. From the bacterial microbiota observed in this study, many biological and epidemiological questions have arisen, especially regarding "how" and "who" are implicated in *I. ventalloi* capacity and vector competence, host adaptation and geographic expansion, that will be interesting to approach in future tick microbiome research studies.

## Availability of data

Raw metagenomic data for the *I. ventalloi* collected at Boschetto Airoldi and Castello Utveggio are deposited in the (Dryad repository doi:10.5061/dryad.7jf48t0).

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2018.11.014.

## References

Abraham, N.M., Liu, L., Jutras, B.L., Yadav, A.K., Narasimhan, S., Gopalakrishnan, V., 2017. Pathogen-mediated manipulation of arthropod microbiota to promote infection. Proc. Natl. Acad. Sci. U.S.A. 114, E781–E790.

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

Allen, B.F., Keesing, F., Ostfeld, R.S., 2003. Effect of forest fragmentation on Lyme disease risk. Conservation Biol. 17, 267–272.

Alvarez, C., Kukutla, P., Jiang, J., Yu, W., Xu, J., 2012. Draft genome sequence of *Pseudomonas* sp. Strain Ag1, isolated from the midgut of the malaria mosquito *A. gambiae*. J. Bacteriol. 194, 5449.

Anderson, J.F., Barthhold, S.W., Magnarelli, L.A., 1990. Infectious but non-pathogenic isolate of *Borrelia burgdorferi*. J. Clin. Microbiol. 28, 2693–2699.

Bell-Sakyi, L., Palomar, A.M., Kazimirova, M., 2015. Isolation and propagation of a *Spiroplasma* sp. From Slovakian *Ixodes ricinus* ticks in *Ixodes* spp. cell lines. Ticks Tick Borne Dis. 6, 601–606.

Beninati, T., Riegler, M., Vilcins, I.-M.E., Sacchi, L., McFadyen, R., Krockenberger, M., Bandi, C., O´Neill, S.L., Lo, N., 2009. Absence of the symbiont *Candidatus* Midichloria mitochondrii of the *Ixodes holocyclus*. FEMS Microbiol. Lett. 299, 241–247.

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.

Breuner, N.E., Dolan, M.C., Replogle, A.J., Sexton, C., Hojgaard, A., Boegler, K.A., Clark, R.J., Eisen, L., 2017. Transmission of *Borrelia miyamotoi sensu lato* relapsing fever group spirochetes in relation to duration of attachment by *Ixodes scapularis* nymphs. Ticks Tick Borne Dis. 8, 677–681.

Brucker, R.M., Bordenstein, S.R., 2013. The capacious hologenome. Zoology 116, 260–261.

Carpi, G., Cagnacci, F., Wittekindt, N.E., Zhao, F., Qi, J., Tomsho, L.P., Drautz, D.I., Rizzoli, A., Schuster, S.C., 2011. Metagenomic profile of the bacterial communities associated with *Ixodes ricinus* ticks. PLoS One 6, e25604.

Chastel, C., Main, A.J., Couatarmanac'h, A., Le Lay, G., Knudson, D.L., Quillien, M.C., Beaucournu, J.C., 1984. Isolation of Eyach virus (*Reoviridae*, Colorado tick fever group) from *Ixodes ricinus* and *I. ventalloi* ticks in France. Arch. Virol. 82, 161–171.

Clay, K., Fuqua, C., 2010. The tick microbiome: diversity, distribution and influence of the internal microbial community for a blood-feeding disease vector. Critical Needs and Gaps in Understand Prevention, Amelioration, and Resolution of Lyme and Other Tick-borne Diseases: the Short-term and Long-term Outcomes. Institute of Medicine Committee on Lyme Disease and Other Tick-Borne Diseases: The State of the Science, Washington DC, USA (Accessed 7 March 2018). http://www.iom.edu/~/media/Files/Activity%20Files/Disease/TickBorne/08-The-Tick-Microbiome.pdf.

Cowdry, E.V., 1925. A group of microorganisms transmitted hereditarily in ticks and apparently unassociated with disease. J. Exp. Med. 41, 817–830.

Clow, K.M., Weese, J.S., Rousseau, J., Jardine, C.M., 2018. Microbiota of field-collected *Ixodes scapularis* and *Dermacentor variabilis* from eastern and southern Ontario, Canada. .Ticks Tick Borne Dis. 9, 235–244.

Dantas-Torres, F., 2015. Climate change, biodiversity, ticks and tick-borne diseases: the butterfly effect. Int. J. Parasitol. Parasites Wild. 4, 452–461.

de la Fuente, J., Villar, M., Cabezas-Cruz, A., Estrada-Peña, A., Ayllón, N., Alberdi, P., 2016. Tick-host-pathogen interactions: conflict and cooperation. PLoS Pathogen 14, e1005488.

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.Mm, Papa, A., Rudenko, N., Villar, M., Alberdi, P., Torina, A., Ayllón, N., Vancova, M., Golovchenko, M., Grubhoffer, L., Caracappa, S., Fooks, A.R., Gortázar, C., Rego, R.M.O., 2017. Tick-pathogen interactions and vector competence: identification of molecular drivers for tick-borne diseases. Front. Cell. Infect. Microbiol. 7, 114.

Duron, O., Binetruy, F., Noël, V., Cremaschi, J., McCoy, K.D., Arnathau, C., Plantard, O., Goolsby, J., Pérez de León, A.A., Heylen, D.J.A., Van Oosten, A.R., Gottlieb, Y., Baneth, G., Guglielmone, A.A., Estrada-Peña, A., Opara, M.N., Zenner, L., Vavre, F., Chevillon, C., 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. Parasitol 135, 485–494.

Eshoo, M.W., Carolanm, H.E., Massire, C., Chou, D.M., Crowder, C.D., Rounds, M.A., Phillipson, C.A., Schutzer, S.E., Ecker, D.J., 2015. Survey of *Ixodes pacificus* ticks in California reveals a diversity of microorganisms and novel and widespread *Anaplasmataceae* species. PLoS One 10, e0135828.

Estrada-Peña, A., Ayllón, N., de la Fuente, J., 2012. Impact of climate trends on tick-borne pathogen transmission. Front. Physiol. 3, 64.

Estrada-Peña, A., Ostfeld, R.S., Peterson, A.T., Poulin, R., de la Fuente, J., 2014. Effects of environmental change on zoonotic disease risk: an ecological primer. Trends Parasitol. 30, 205–214.

Frith, M.C., Hamada, M., Horton, P., 2010a. Parameters for accurate genome alignment. BMC Bioinformatics 11 (80).

Frith, M.C., Wan, R., Horton, P., 2010b. Incorporating sequence quality data into alignment improves DNA read mapping. Nucleic Acids Res. 38, e100.

Greay, T.L., Gofton, A.W., Paparini, A., Ryan, U.M., Oskam, C.L.M., Irwin, P.J., 2018. Recent insights into the tick microbiome gained through next-generation sequencing. Parasit. Vectors 11 (12).

Henning, K., Greiner-Fischer, S., Hotzel, H., Ebsen, M., Theegarten, D., 2006. Isolation of *Spiroplasma* sp. from an *Ixodes* tick. Int. J. Med. Microbiol. 296, 157–161.

Hernández-Jarguín, A., Díaz-Sánchez, S., Villar, M., de la Fuente, J., 2018. Integrated metatranscriptomics and metaproteomics for the characterization of bacterial microbiota in unfed *Ixodes ricinus*. Ticks Tick Borne Dis. 9, 1241–1251.

Huber, D., Rei, I., Duvnjank, S., Jurković, D., Lukačevic, D., Pilat, M., Beck, A., Mihaljević, Z., Vojta, L., Polkinghorne, A., Beck, R., 2017. Molecular detection of *Anaplasma platys*, *Anaplasma phagocythophilum* and *Wolbachia* sp. but not *Ehrlichia canis* in Croatian dogs. Parasitol. Res. 116, 3019–3026.

Hunter, D.J., Torkelson, J.L., Bodnar, J., Mortazavi, B., Laurent, T., Deason, J., Zhong, J., 2015. The rickettsia endosymbiont of *Ixodes pacificus* contains all the genes of *de novo* folate biosynthesis. PLoS One 10, e0144552.

Jameson, L.J., Medlock, J.M., 2011. Tick surveillance in Great Britain. Vector Borne Zoonotic Dis. 11, 403–412.

Jongejan, F., Uilenberg, G., 2004. The global importance of ticks. Parasitology 129, S3–S14.

Kiełbasa, S.M., Wan, R., Sato, K., Horton, P., Frith, M.C., 2011. Adaptive seeds tame genomic sequence comparison. Genome Res. 21, 487–493.

Kurilshikov, A., Livanova, N.N., Fomenko, N.V., Tupikin, A.E., Rar, V.A., Kabilov, M.R., Livanov, S.G., Tikunova, N.V., 2015. Comparative metagenomic profiling of symbiotic bacterial communities associated with *Ixodes persulcatus, Ixodes pavlovskyi* and *Dermacentor reticulatus* ticks. PLoS One 10, e0131413.

Latrofa, M.E., Gianelli, A., Persichetti, M.F., Pennissi, M.G., Solano-Gallego, L., Brianti, E., Parisi, A., Wall, R., Dantas-Torres, F., Otranto, D., 2017. *Ixodes ventalloi:* morphological and molecular support for species integrity. Parasitol. Res. 116, 251–258.

Lo, N., Beninati, T., Sacchi, L., Bandi, C., 2006. An alpha-proteobacterium invades the mitochondria of the tick *Ixodes ricinus*. In: Bourtzis, K., Miller, T.A. (Eds.), Insect Symbiosis. Taylor & Francis, Boca Raton, Florida, pp. 25–37.

Lo, W.S., Gasparich, G.E., Kuo, C.H., 2015. Found and lost: the fates of horizontally acquired genes in arthropod-symbiotic *Spiroplasma*. Genome Biol. Evol. 8, 2458–2472.

Lysyk, T.J., Kalischuk-Tymensen, L., Selinger, L.B., Lancaster, R.C., Wever, L., Cheng, K.J., 1999. Rearing stable fly larvae (Diptera: muscidae) on an egg yolk medium. J. Med. Entomol. 36, 382–388.

Manilla, G., 1998. Fauna dÍtalia. Acari: *Ixodidae*. Edizioni Calderini, Bologna, Italy.

Margos, G., Vollmer, S.A., Ogden, N.H., Fish, D., 2011. Population genetics, taxonomy, phylogeny and evolution of *Borrelia burgdorferi sensu lato*. Infect. Genet. Evol. 11, 1545–1563.

Margos, G., Piesman, J., Lane, R.S., Ogden, N.H., Sing, A., Straubinger, R.K., Fingerle, V., 2014. Borrelia kurtenbachii sp. nov., a widely distributed member of the *Borrelia burdogferi* sensu lato species complex in North America. Int. J. Syst. Evol. Microbiol. 64, 128–130.

Moutailler, S., Valiente Moro, C., 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.

Nakao, R., Abe, T., Nijhof, A.M., Yamamoto, S., Jongejan, F., Ikemura, T., Sugimoto, C., 2013. A novel approach, based on BLSOMs (Batch Learning Self-Organizing Maps), to the microbiome analysis of ticks. ISME J. 7, 1003–1015.

Narasimhan, S., Rajeevan, N., Liu, L., Zhao, Y.O., Heisig, J., Pan, J., Eppler-Epstein, R., Deponte, K., Fish, D., Fikrig, E., 2014. Gut microbiota of the tick vector *Ixodes scapularis* modulate colonization of the Lyme disease spirochete. Cell Host Microbe 15, 58–71.

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

Niels, O.V., Boulanger, N., Spitzen, J., 2018. Impact of skin microbiome on attractiveness to arthropod vectors and pathogen transmission. In: Boulanger, N. (Ed.), Skin and Arthropod Vectors. Elsevier, pp. 55–81.

Njam, N.A., Silñaghi, C., Bell-Sakyio, L., Pfister, K., Passos, L.M., 2012. Detection of bacteria related to *Candidatus* Midichloria mitochondrii in tick cell lines. Parasitol. Res. 110.

Otranto, D., Dantas-Torres, F., Giannelli, A., Latrofa, M.S., Cascio, A., Cazzin, S., Ravagnan, S., Montarsi, F., Zanzani, S.A., Manfredi, M.T., Capelli, G., 2014. Ticks infesting humans in Italy and associated pathogens. Parasit. Vectors 7, 32.

Petney, T.N., Beichel, E., Maiwald, M., Hassler, D., 1996. *Ixodes ventalloi*: a new tick record for Germany. Appl. Parasitol. 37, 96–98.

Pennisi, M.G., Persichetti, M.F., Serrano, L., Altet, L., Reale, S., Gulotta, L., Solano-Gallego, L., 2015. Parasit. Vectors 8, 512.

Portillo, A., Santos, A.S., Santibañez, S., Pérez-Martínez, L., Blanco, J.R., Ibarra, V., Oteo, J.A., 2005. Detection of a non-pathogenic variant of *Anaplasma phagocytophilum* in *Ixodes ricinus* from La Rioja. Ann. N. Y. Acad. Sci. 1063, 333–336.

Qiu, Y., Nakao, R., Ohnuma, A., Kawamori, F., Sugimoto, C., 2014. Microbial population analysis of the salivary glands of ticks; a possible strategy for the surveillance of bacterial pathogens. PLoS One 9, e103961.

Raimondo, F.M., Venturella, G., 1993a. Lineamenti fisici e biogeografici del promontorio di Monte Pellegrino (Palermo). Quad. Bot. Amb. Appl. 4, 7–11.

Raimondo, F.M., Venturella, G., Ilardi, E.V., 1993b. Carta forestable del promontorio di Monte Pellegrion, (Palermo). Quad. Bot. Amb. Appl. 4, 145–152.

Rynkiewicz, E., Hemmerich, C., Rusch, D.B., Fuqua, C., Clay, K., 2015. Concordance of bacterial communitites of two tick species and blood of their shared rodent host. Mol. Ecol. 14, 2566–2579.

Salter, S.J., Cox, M.J., Turek, E.M., Calus, S.T., Cookson, W.O., Moffatt, M.F., Turner, P., Parkhill, J., Loman, N.J., Walker, A.W., 2014. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol. 2014 (12), 87.

Santos Dias, J.A.T., Santos-Reis, M., 1989. A carrac¸a *Ixodes ventalloi* Gil Collado, 1936 como principal ectoparasitade uma populacao de doninhas (*Mustela nivalis* Linnaeus, 1766) em Portugal. Garcia de Orta, Serie de Zoologia. 14, 35–50.

Santos, A.S., Santos-Silva, M.M., Almeida, V.C., Bacellar, F., Dumlert, J.S., 2004. Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* ticks (Acari: Ixodidae) from Madeira island and Setúbal district, Mailand Portugal. Emerg. Infect. Dis. 10, 1643–1648.

Santos-Silva, M.M., Sousa, R., Santos, A.S., Melo, P., Encarnação, V., Bacellar, F., 2006. Ticks parasitizing wild birds in Portugal: detection of *Rickettsia aeschlimannii*, *R. helvetica and R. m* and *R. massiliae*. Exp. Appl. Acarol. 39, 331–338.

Santos-Silva, M.M., Beati, L., Santos, A.S., De Sousa, R., Núncio, M.S., Melo, P., Bacelllar, F., 2011. The hard-tick fauna of mainland Portugal (Acari: Ixodidae): an update on geographical distribution and known associations with host and pathogens. Exp. Appl. Acarol. 55, 85–121.

Sevellec, M., Derome, N., Bernatchez, L., 2018. Holobionts and ecological speciation: the intestinal microbiota of lake whitefish species pairs. Microbiome 6, 47.

Sui, S., Yang, Y., Sun, Y., Wang, X., Wang, G., Shan, G., et al., 2017. On the common bacterial flora of *Ixodes persulcatus* (Taiga tick). PLoS One 12, e0180150.

Surano, N., Gianguzzi, L., Raimondo, F.M., 1993. Carta della vegetazione del promontorio di Monte Pellegrino (Palermo). Quad. Bot. Amb. Appl. 4, 139–144.

Taylor, M., Meddiannikov, O., Raoult, D., Greub, G., 2015. Endosymbiotic bacteria associated with nematodes, ticks and amoebae. FEMS Immunol. Med. Microbiol. 64, 21–31.

Tomassone, L., Grego, E., Auricchio, D., Lori, A., Giannini, F., Rambozzi, L., 2013. Lyme borreliosis spirochetes and spotted fever group rickettsiae in *Ixodid* ticks from Pianosa island, Tuscany Archipielago, Italy. Vector-Borne Zoonotic Dis. 13.

Torina, A., Blanda, V., Blanda, M., Auteri, M., La Russa, F., Scimeca, S., D´ Agostino, R., Disclafani, R., Villari, S., Currò, V., Caracappa, S., 2018. A geographical information system based approach for integrated strategies of tick surveillance and control in the peri-urban natural reserve of Monte Pellegrino (Palermo, Southern Italy). Int. J. Environ. Res. Public Health 15, 404.

Tully, J.G., Rose, D.L., Yunker, C.E., Cory, J., Whitcomb, R.F., Williamson, D.L., 1981. Helical mycoplasmas (spiroplasmas) from *Ixodes* ticks. Science. 212, 1043–1045.

Van Treuren, W., Ponnusamy, L., Brinkerhoff, R.J., Gonzalez, A., Parobek, C.M., Juliano, J.J., Andreadis, T.G., Falco, R.C., Ziegler, L.B., Hathaway, N., Keeler, C., Emch, M., Bailey, J.A., Roe, R.M., Apperson, C.S., Knight, R., Meshnick, S.R., 2015. Variation in the microbiota of *Ixodes* ticks with regard to geography, species, and sex. Appl. Environ. Microbiol. 81, 6200–6209.

Vayssier-Taussat, M., Moutailler, S., Michelet, L., Devillers, E., Bonnet, S., Cheval, J., Hébert, C., Eloit, M., 2013. Next-generation sequencing uncovers unexpected bacterial pathogens in ticks in western Europe. PLoS One 8, e81439.

Zolnik, C.P., Prill, R.J., Falco, R.C., Daniels, T.J., Kolokotronis, S.O., 2016. Microbiome changes through ontogeny of a tick-pathogen vector. Mol. Ecol. 25, 4963–4977.