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Microbial associates of blood-sucking arthropods and other animals: Relevance to their physiology, ecology and evolution

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Published in
Frontiers in Microbiology

ISSN 1664-8714
ISBN 978-2-8325-3211-9
DOI 10.3389/978-2-8325-3211-9

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Microbial associates of blood-sucking arthropods and other animals: Relevance to their physiology, ecology and evolution

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Citation

Fukatsu, T., Gottlieb, Y., Graf, J., eds. (2023). *Microbial associates of blood-sucking arthropods and other animals: Relevance to their physiology, ecology and evolution*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-3211-9

Table of contents

frontiers | Frontiers in Microbiology

TYPE Editorial
PUBLISHED 26 July 2023
DOI 10.3389/fmicb.2023.1256275

OPEN ACCESS

EDITED AND REVIEWED BY
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RECEIVED 10 July 2023
ACCEPTED 17 July 2023
PUBLISHED 26 July 2023

CITATION
Fukatsu T, Gottlieb Y, Duron O and Graf J (2023) Editorial: Microbial associates of blood-sucking arthropods and other animals: relevance to their physiology, ecology and evolution. *Front. Microbiol.* 14:1256275. doi: 10.3389/fmicb.2023.1256275

Editorial: Microbial associates of blood-sucking arthropods and other animals: relevance to their physiology, ecology and evolution

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KEYWORDS

blood-feeding, insect, tick, mite, crustacean, leech, microbiome, B vitamin

Editorial on the Research Topic
Microbial associates of blood-sucking arthropods and other animals: relevance to their physiology, ecology and evolution

Lice, bed bugs, ticks, leeches, and other tiny blood-sucking crawling creatures are regarded as nasty vampires, causing itches, eliciting disgusting feeling, vectoring human and animal diseases, and thereby bringing about medical, health, hygienic and mental problems in human societies (Lehane, 2005). Besides the microbial pathogens they carry and transmit, unique microorganisms are associated with them and affect their physiology, ecology, and other biological aspects in a variety of ways (Rio et al., 2016; Husnik, 2018). For example, their food, vertebrate blood, is certainly nutrition-rich, but devoid of some important nutrients like B vitamins. Hence, many blood feeders possess specialized organs called bacteriomes for hosting vitamin-provisioning symbionts (Buchner, 1965), which enable them to thrive only on the blood meal (Duron and Gottlieb, 2020). Fully engorged blood feeders exhibit a challenging gut environment with plenty of proteins, iron, heme and antimicrobial components such as antibodies and complements, which may foster unique gut microbiome (Sterkel et al., 2017).

Owing to recent development of high-throughput DNA sequencing technologies, our knowledge of the microbiomes associated with these blood-sucking invertebrates, which must be connected to their unique feeding habit and physiology, has been growing rapidly. Hence, this Research Topic "*Microbial Associates of Blood-Sucking Arthropods and Other Animals: Relevance to Their Physiology, Ecology and Evolution*" is aimed to provide a forum for new findings emerging in this research field. In total, nine articles and two reviews are compiled, which showcase the microbial associates of a diverse array of blood-feeding invertebrates including lice (Insecta: Psocodea), tsetse flies (Insecta: Diptera), fleas (Insecta: Siphonaptera), ticks (Arachnida: Ixodida) and mites (Arachnida: Mesostigmata) from

the terrestrial ecosystem, and *Elthusa* and *Nerocila* (Crustacea: Isopoda), *Lernanthropus* (Crustacea: Copepoda) and fish leeches (Hirudinea: Piscicolidae) from the marine ecosystem.

Sucking lice (Psocodea: Anoplura) live on vertebrate blood as the sole food source throughout their life cycle (Durden and Musser, 1994), many of which possess specialized symbiotic organs for harboring specific symbiotic bacteria (Ries, 1931; Buchner, 1965). Both histological inspection and molecular phylogenetic survey revealed that their symbiotic organs and associated bacterial symbionts are strikingly diverse among different lice lineages and likely of independent evolutionary origins (Hypša and KriŽek, 2007; Boyd and Reed, 2012). In this Research Topic, three articles dealt with louse-associated symbiotic bacteria. Nishide, Oguchi, et al., investigated the endosymbiotic microbiota of the boar louse *Haematopinus apri,* identified a primary endosymbiont clade associated with the boar, swine and cattle lice, and designated it as "*Candidatus* Haematopinicola symbiotica". Říhová et al. screened and assembled the metagenomic data of the chipmunk lice *Neohaematopinus* spp. and identified a genome-reduced endosymbiont designated as "*Candidatus* Lightella neohaematopini". Doña et al. surveyed the microbiota associated with the seal louse *Echinophthirius horridus*, which uncovered diverse bacterial associates but failed to identify principal symbiotic bacteria. These reports highlight the dynamic evolutionary trajectories of the louse-microbe endosymbiotic associations entailing multiple and independent gains and losses.

Tsetse flies (Diptera: Glossinidae) are obligatory blood feeders distributed in sub-Saharan Africa, where they vector devastating human and animal pathogens *Trypanosoma* spp. (Krafsur, 2009). Tsetse flies are associated with a vitamin-provisioning primary symbiont *Wigglesworthia glossinidia*, a commensal bacterial associate *Sodalis glossinidius*, and a facultative endosymbiont *Wolbachia pipientis* (Aksoy, 2000). In the Research Topic, Lee et al. reviewed the current understanding of tsetse-microbe molecular interactions, with particular focus on recently accumulating knowledge about possible involvement of DNA methylation and microRNAs.

Fleas (Siphonaptera) are obligatory blood feeders of mammals and birds as adults, and notorious for vectoring *Yersinia pestis* and other pathogens (Bitam et al., 2010). Probably because their larvae live on organic debris without blood feeding, no obligatory microbial symbionts have been known from fleas, whereas diverse facultative bacterial associates have been detected, as Dong et al. identified *Wolbachia*, *Rickettsia* and *Bartonella* as the major bacterial associates of the fleas *Oropsylla silantiewi* and *Callopsylla dolabris* from Himalayan marmots. In the flea *Synosternus cleopatrae* from desert rodents, it was reported that, interestingly, *Wolbachia* infection is fixed in females but lacking or partial in males (Flatau et al., 2018). In this Research Topic, Flatau et al. treated *S. cleopatrae* with tetracycline and compared the life history parameters of *Wolbachia*-infected fleas with those of *Wolbachia*-free fleas, but no significant differences were detected between them.

Ticks (Ixodida: Ixodea) are obligatory blood feeders of terrestrial vertebrates including mammals, birds, reptiles and amphibians (Anderson and Magnarelli, 2008). Conventionally, ticks have been regarded as vectors of *Rickettsia*, *Coxiella* and other pathogens causing human and animal diseases (de la Fuente et al., 2008), but now it is widely recognized that ticks commonly host non-pathogenic, either commensalistic or mutualistic, microbial associates allied to *Coxiella*, *Rickettsia*, *Francisella*, *Midichloria*, *Wolbachia* and others (Bonnet et al., 2017). In this Research Topic, Hussain et al. reviewed such tick-microbe symbiotic continuum spanning from pathogens through commensals to mutualists. Dong et al. detected *Anaplasma*, *Wolbachia* and *Ehrlichia* as the major bacterial associates of the tick *Haemaphysalis qinghaiensis* from Himalayan marmots. Militzer et al. analyzed the effects of artificial feeding and antibiotic treatment on microbiome composition and fecundity of the tick *Ixodes ricinus* associated with *Midichloria*, *Rickettsia* and *Spiroplasma*.

The poultry red mite *Dermanyssus gallinae* (Mesostigmata: Dermanyssidae) is a blood sucking avian ectoparasite that often causes significant economic damage on poultry production (Sparagano et al., 2014). A previous study identified *Bartonella*, *Cardinium*, *Wolbachia* and *Rickettsiella* in European populations of *D. gallinae* (Hubert et al., 2017). In this Research Topic, Price et al. detected *Rickettsiella* from all 63 samples of *D. gallinae* derived from 63 localities across 15 European countries, and determined the 1.9 Mbp *Rickettsiella* genome that retains the synthetic pathways for thiamine (= vitamin B1), riboflavin (= vitamin B2) and pyridoxine (= vitamin B6). By contrast, Nishide, Sugimoto, et al., reported that, from 144 samples of *D. gallinae* collected from 18 poultry farms in Japan, *Bartonella*, *Cardinium*, *Wolbachia* and *Tsukamurella* were detected as major bacterial components, but *Rickettsiella* was not detected at all. These reports uncovered strikingly different microbiota across European and Japanese populations of *D. gallinae*. At present, whether *Rickettsiella* is the vitamin-provisioning primary symbiont of *D. gallinae* or not is elusive and to be established in future studies.

Finally, as the highlight of this Research Topic, Goffredi et al. reported the microbiomes of marine obligatory blood feeders that have been little investigated previously: fish ectoparasitic isopods *Elthusa vulgaris* and *Nerocila californica* (Isopoda: Cymothoidae); a fish ectoparasitic copepod *Lernanthropus latis* (Copepoda: Lernanthropidae); and fish leeches *Pterobdella occidentalis*, *Ostreobdella californiana*, and *Branchellion lobata* (Hirudinea: Piscicolidae). Interestingly, all the marine blood suckers exhibited peculiar gut microbiomes characterized by relatively low diversity dominated by *Vibrio* species.

In conclusion, the Research Topic presents an impressive overview of the current research coverage on the diversity of microbial symbioses among blood-sucking arthropods and other invertebrates. These reports significantly broaden our knowledge as to what types of microbiomes are associated with the obligatory blood feeders, and highlight the untouched research fields represented by, for example, marine obligatory blood feeders and their biological and functional aspects, as promising targets for future studies. It has been widely accepted that symbiotic interactions with microorganisms are essential for the ecology of insects, ticks, leeches and other invertebrates with obligatory blood feeding habit. Diverse bacterial lineages have independently evolved functional interactions with the obligatory blood feeders, but, notably, all converge to an analogous biochemical feature of vitamin provisioning.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 March 2021
doi: 10.3389/fmicb.2021.649248

Wolbachia Endosymbionts of Fleas Occur in All Females but Rarely in Males and Do Not Show Evidence of Obligatory Relationships, Fitness Effects, or Sex-Distorting Manipulations

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OPEN ACCESS

Edited by:
Olivier Duron, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Daisuke Kageyama, National Agriculture and Food Research Organization (NARO), Japan
Jianmin Zhong, Humboldt State University, United States

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Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *04 January 2021*
Accepted: *15 February 2021*
Published: *12 March 2021*

Citation:
Flatau R, Segoli M and Hawlena H (2021) Wolbachia Endosymbionts of Fleas Occur in All Females but Rarely in Males and Do Not Show Evidence of Obligatory Relationships, Fitness Effects, or Sex-Distorting Manipulations. Front. Microbiol. 12:649248. doi: 10.3389/fmicb.2021.649248

The widespread temporal and spatial persistence of endosymbionts in arthropod host populations, despite potential conflicts with their hosts and fluctuating environmental conditions, is puzzling. Here, we disentangled three main mechanisms that are commonly proposed to explain such persistence, namely, obligatory relationships, in which the host is fully dependent on its endosymbiont, fitness advantages conferred by the endosymbiont, and reproductive manipulations imposed by the endosymbiont. Our model system reflects an extreme case, in which the *Wolbachia* endosymbiont persists in all female flea hosts but rarely in male ones. We cured fleas of both sexes of *Wolbachia* but found no indications for either lower reproduction, offspring survival, or a change in the offspring sex ratio, compared to *Wolbacia*-infected fleas. These results do not support any of the suggested mechanisms. We highlight future directions to advance our understanding of endosymbiont persistence in fleas, as well as in other model systems, with extreme sex-differences in endosymbiont persistence. Insights from such studies are predicted to shed light on the evolution and ecology of arthropod-endosymbiont interactions in nature.

Keywords: antibiotic treatment, arthropod symbiosis, experiment, fitness, fleas, persistence mechanisms, reproductive manipulations, *Wolbachia*

INTRODUCTION

Highly prevalent and dense endosymbiont populations that persist in arthropod host populations, despite potential conflicts with their hosts and fluctuating environmental conditions, are widespread in nature (Feldhaar, 2011). Several mechanisms have been proposed to explain such high persistence levels. One possible explanation is related to the tendency of the endosymbiont-host relationships to evolve into a full dependence of the host on the endosymbiont for its survival and reproduction in the form of **obligatory relationships** (Moran et al., 2008; Ferrari and Vavre, 2011). However, endosymbionts may promote persistence even when the host is not fully dependent on them, for example, by providing **fitness advantages** to the

host, e.g., nutrient supplementation or protection from enemies (Feldhaar, 2011; Su et al., 2013; Cao et al., 2019). A third possible group of mechanisms is related to **reproductive manipulations** imposed by the endosymbiont on the host's reproduction to enhance its own transmission (Werren et al., 2008). Considering the important function that endosymbionts serve in determining the structure and performance of natural communities and their potential uses in biological control (Ahantarig and Kittayapong, 2011), understanding the relative roles that these mechanisms play in the persistence of arthropod-endosymbiont systems and identifying new potential mechanisms constitute major goals with applied aspects.

Wolbachia is among the most widespread bacterial endosymbionts in nature, infecting various arthropod and nematode species (Werren et al., 2008; Gerth et al., 2014). Some strains of *Wolbachia* exhibit **obligatory relationships** with their host, providing them with essential functions (Nikoh et al., 2014). For example, in bedbugs, *Wolbachia* is essential for the host's growth and reproduction by providing B vitamins, which are deficient in their blood-based diet (Hosokawa et al., 2010). Other *Wolbachia* strains are involved in facultative relationships with their hosts, providing their host with **fitness advantages**, thereby enhancing their spread in the host population (Dean, 2006; Moran et al., 2008). For example, *Wolbachia* may enhance their host's fitness through protection against pathogens (Hedges et al., 2008; Teixeira et al., 2008) and through nutritional advantages (Brownlie et al., 2009).

Finally, *Wolbachia* can spread and persist in the host population by manipulating their host's reproduction to enhance the fitness of infected females. Such **reproductive manipulations** may include: (i) male-killing (MK), where infected males are eliminated, resulting in reduced competition for the surviving female progeny (Hurst and Jiggins, 2000; Werren et al., 2008); (ii) the induction of parthenogenesis (IP), where females produce daughters asexually (Ma and Schwander, 2017); (iii) the feminization of genetic males (MF) allowing them to produce eggs (Narita et al., 2011); and (iv) cytoplasmic incompatibility (CI), where the offspring of infected males and uninfected females fail to develop (or may develop into males in the case of arthropods with haplodiploid sex determination), thereby providing a reproductive advantage to infected females (Mouton et al., 2005; Werren et al., 2008).

To better understand endosymbiont persistence patterns and mechanisms in their host, we studied the fitness effects of the endosymbiont *Wolbachia* on their flea host *Synosternus cleopatrae*, which infests desert rodents. This system provides an important opportunity to test the universality of the above-suggested persistence mechanisms, as several lines of evidence suggest that it might differ from most previously documented cases. First, this is one of a few cases in which extreme sex-specific differences in endosymbiont persistence were documented over both time and space (see also Lo et al., 2006; Richardson et al., 2019). In fact, in natural populations, all female fleas in all sampling locations and times possess *Wolbachia* at high loads ($3 \times 10^5 \pm 2 \times 10^5$), whereas from 0 to 54% of the males possess *Wolbachia* at a detectable level, with low infection loads ($7 \times 10^3 \pm 1 \times 10^4$; Cohen et al., 2015; Flatau et al., 2018). Such extreme bias in the endosymbiont persistence pattern suggests that the two sexes are prone to divergent selection pressures (Richardson et al., 2019). Second, the near absence of *Wolbachia* in male fleas also excludes the possibility of *Wolbachia* being involved in **obligatory relationships**, at least regarding the males. Third, a previous study suggested the possibility of some negative fitness effects of *Wolbachia* on female fleas, as females with a relatively higher density of *Wolbachia* had a lower fitness, negating the possibility of *Wolbachia* spread in the population *via* **fitness advantages**. Nevertheless, these results were observational and could have been confounded with the female physiological age (Flatau et al., 2018). Finally, high *Wolbachia* persistence in females is also not likely to be explained by the occurrence of strong **reproductive manipulation** because the host species does not exhibit a female-biased sex ratio, reducing the possibility of strong sex-distorting manipulations such as MK, IP, or MF (Werren et al., 2008). In addition, the low occurrence and extremely low density of *Wolbachia* in male fleas (Flatau et al., 2018) suggest the lack of strong CI (but see Richardson et al., 2019).

To disentangle the occurrence of these effects, we cured fleas of both sexes of *Wolbachia* and assessed the reproductive success of female fleas in relation to their physiological age and *Wolbachia* presence. A failure of cured female fleas to survive and reproduce would support an **obligatory relationship**. A reduction in the fitness-correlative traits of the cured fleas would support the **fitness advantage** mechanism. A higher proportion of female offspring in *Wolbachia*-infected, compared to cured, fleas would support the occurrence of a **reproductive manipulation** inducing a female-biased sex ratio (MK, IP, or MF). In addition, we characterized the location of these endosymbionts within female fleas, as this may hint at their function. For example, endosymbiont occurrence in the gut and gut appendages may suggest a nutritional role (Ben-Yosef et al., 2020; Reis et al., 2020).

MATERIALS AND METHODS

Experimental Design

Larval *S. cleopatrae* fleas were randomly assigned to one of two treatment groups. One group received tetracycline antibiotics as a supplement to their food, and a control group received the same food except for the antibiotic supplementation (**Figure 1A**). Then, the adult fleas emerging from both treatment groups were reared separately on rodents for an additional three generations (150 days) without antibiotics (**Figure 1B**). The goal of this second stage was to reduce the direct effect of the antibiotics on the fleas and to allow the fleas to restore their natural bacterial community, excluding *Wolbachia* that cannot be acquired from the environment (Werren et al., 2008). The only other potential maternal transmitted bacteria that have been detected in *S. cleopatrae* fleas belong to *Rickettsia*. However, it was detected in one male out of 59 male and female fleas in one study (Cohen et al., 2015) and in one of 91 pools of fleas in another (Rzotkiewicz et al., 2015), suggesting that *Wolbachia* is the main intra-cellular endosymbiont found in these female fleas. For the third experimental stage, each treatment group was randomly divided into six subgroups

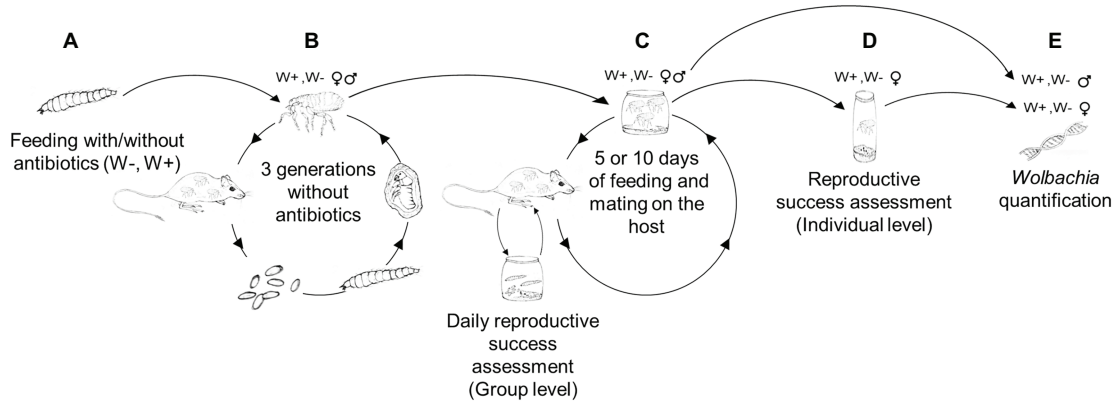

FIGURE 1 | Experimental design. **(A)** Larval *Synosternus cleopatrae* fleas were randomly assigned to two treatment groups: a group that received antibiotics as a supplement to their food, *Wolbachia*-free (W^-) and a control (W^+) group. **(B)** Fleas from the two groups were reared on rodents for three additional generations without the antibiotic supplement. **(C)** Each treatment group was randomly divided into six subgroups, each subjected to either 5 or 10 days of feeding and mating. Specifically, the fleas were allowed to feed daily for 2 h on rodents and then collected and incubated overnight in separate boxes, where the average daily reproductive success of the group was assessed. **(D)** At the end of the three experimental stages, all female fleas were individually subjected to reproductive success assessments. **(E)** Then, a subset of fleas was subjected to *Wolbachia* quantification.

(15 females and 15 males per group). Each subgroup was then subjected to either 5 or 10 days of feeding and mating. Specifically, the fleas were allowed to feed and mate on rodents for 2 h daily. The fleas were then collected and incubated overnight in separate boxes, where the average daily reproductive success of the entire subgroup was assessed (i.e., group level; **Figure 1C**). At the end of the third experimental stage, female fleas were collected into separate plastic vials, where the individual parent females were allowed to lay eggs for 24 h. Then, the vials were subjected to reproductive success assessments (i.e., individual level; **Figure 1D**), and a subset of the parental and offspring fleas were subjected to *Wolbachia* assessments (**Figure 1E**). Below, we detail each of the experimental stages.

Establishment of the *Wolbachia*-Free (W^-) and *Wolbachia*-Control (W^+) Treatment Groups

One thousand *S. cleopatrae* fleas were randomly collected from a laboratory colony (courtesy of Prof. Krasnov, Ben-Gurion University). This flea colony has been maintained for 17 years (124 generations) on laboratory-reared gerbils, during which the genetic diversity was sustained through an annual mixing with wild fleas. Notably, although some genomic differences between laboratory and wild fleas are possible, the observed pattern of high *Wolbachia* prevalence (100%) and density in females compared to males is consistent in both populations (Flatau et al., 2018). These fleas were allowed to feed and mate on 10 *Gerbillus andersoni* rodents and to oviposit eggs in the rodent sterile sand bedding for 7 days. Then, the sand from all cages was mixed and equally divided into 10 20 cm × 15 cm × 10 cm boxes that were incubated at 25 ± 1°C and 95 ± 3% humidity in a growth chamber (I-41Nl, Percival Scientific, Inc.). Five of the boxes were randomly assigned to the antibiotic treatment, and the other five were used as controls. For the next 12 days, all boxes were supplemented every 4 days, with 10 g of larvae food mixed in the sand. In the treatment boxes, the food was mixed with an increasing amount of tetracycline powder (100, 200, and 300 mg). Since the tetracycline is fully degraded after 4 days at our incubation conditions, whereas the amount of food consumed by the flea larvae is negligible relative to the supplemented amount, this feeding regime resulted in a concentration of approximately 10 mg tetracycline per 1 g of food throughout the feeding period. Such concentration was found to be most efficient in *Wolbachia* clearance during preliminary trials comparing various combinations of antibiotic types and concentrations (Flatau, unpublished data). The food supplement was composed of 94% dry bovine blood, 5% millet flour and ground local vegetation, and 1% freshly ground rodent excrement. This supplement provides *ad libitum* food for the flea larvae, thus reducing intraspecific competition and increasing larval survival rates (Khokhlova et al., 2014).

Feeding and Mating of Adult Fleas on Rodents

Daily feeding and mating of adult fleas took place on laboratory-reared *G. andersoni* rodents. Fleas were fed in groups of 30 (0.5 sex ratio) to reduce their negative impact on rodents and intraspecific competition among fleas (Hawlena et al., 2006, 2007). To prevent the rodent from grooming, which may harm the fleas, we placed each rodent inside a metal net tube that restricted their movement. Each flea group was fed for 2 h, and then the fleas were brushed off the rodent over a white plastic pan until all were recovered. Exceptions included four cases in which the flea could not be found, and hence its host was not reused, and the 21 cases in which the flea died during feeding. This feeding period is considered sufficient for the fleas to consume a full blood meal and mate (Khokhlova, personal comment). The fleas from each group were incubated

overnight at 25 ± 1°C and 95 ± 3% in separately ventilated 250-ml plastic boxes embedded with 5 ml of sterile sand, where they could lay eggs. These plastic boxes were later used for the daily reproductive success assessment at the group level. Flea subgroups were rotated daily between individual rodents to maximize the number of rodents encountered by each group.

Reproductive Success Assessment

The reproductive success of female fleas was evaluated at both the group and the individual female levels. For the group-level assessment, we used the 250-ml plastic boxes, in which the female fleas were incubated in groups overnight and where they laid eggs following the daily feeding and mating stage (**Figure 1C**). This procedure was repeated every day with a new plastic box, while the previous box was kept in incubation for 50 days, until all offspring have emerged. The boxes were filled with 5 ml of sterile sand and supplemented with 1.25 ml of antibiotic-free larval food (see "establishment of the *Wolbachia*-free and control groups") and incubated at 25 ± 1°C and 95 ± 3% relative humidity. After each box reached 50 days of incubation, all emerged offspring were counted and sexed, and the daily average offspring number per female and offspring sex ratio of each group were quantified.

For the individual level assessment, 43 *Wolbachia*-free and 39 control female fleas were sampled on day 5 of the third experimental stage, following feeding. Then, 45 *Wolbachia*-free and 40 control female fleas were sampled on day 10 of the third experimental stage, following feeding. These 167 female fleas were placed into individual ventilated 10-ml plastic vials with 1 ml of sterile sand. Vials were supplemented with 0.25 ml of antibiotic-free larval food and incubated. From day 30 and on, the vials were monitored daily for newly emerged offspring. Emerged adults were collected into individual 1.5-ml Eppendorf tubes filled with 0.05 ml of sterile sand and incubated at 25 ± 1°C and 95 ± 3% relative humidity, where they were monitored daily until their death.

The emerged adults were not fed, and hence the number of days until their death was used to quantify their survival rate under starvation. Upon death, each offspring was stored in 70% ethanol at −80°C until it was sexed, measured, and, in some cases, subjected to *Wolbachia* quantification (stage E). For each offspring, we measured the two tibias (three repeated measurements per tibia), and the mean between them was raised to the power of three and used as an approximation of each offspring's body size (Messika et al., 2017). Tibia measurements were performed with a stereomicroscope (SMZ18) equipped with a digital camera (DS-Fi2) and with the aid of the program NIS Elements Documentation (Nikon Instruments Inc.). Accordingly, we estimated the reproductive success (RS) of individual females using an integrated index, following Flatau et al. (2018):

$$\text{Equation 1: } RS = \sum_{i}^{NF}\left(BS_F \times PS_F\right) + \sum_{I}^{NM}\left(BS_M \times PS_M\right), \text{ where}$$

NF and NM are the total numbers of female and male offspring, respectively, BSF and BSM are the body sizes of female and male offspring, respectively, and PSF and PSM are the survival probabilities of female and male offspring under starvation, respectively, estimated from the Kaplan-Meier survival analysis (packages "survival" and "survminer"; R Core Team, 2020).

Wolbachia Quantification

DNA was extracted from all-female parent fleas, 40 of the parent males (10 per treatment-age combination), and 26 of the female offspring (13 per treatment group), using the QIAGEN DNeasy Blood and Tissue Kit and was subjected to quantitative polymerase chain reaction (qPCR) tests following the primers and conditions described in Flatau et al. (2018). In each extraction session, a negative control was added in which all the reagents were added to double distilled water instead of fleas. These control extracts were included in the qPCR runs and none of them were amplified.

FISH Analyses

Fluorescence *in situ* hybridizations (FISH) analyses were used to confirm antibiotic supplementation efficiency and locate the *Wolbachia* cells in control fleas. Specifically, 20 female and 20 male fleas (10 per treatment) were immobilized at −20°C for 2 min and then dissected, and parts of their digestive systems (saliva glands, Malpighian tubules, and midguts) and reproductive organs (ovaries, spermathecae, and the male genitalia) were fixed, marked, and scanned, following Kliot et al. (2014). *Wolbachia*-free fleas and flea samples without probes were used as negative controls.

Data Analysis

To test the effect of *Wolbachia* on flea reproductive success at the group level, we performed a generalized linear model, with the treatment (control "W^+" or *Wolbachia*-free "W^-"), female physiological age (covariant; 1–9 days), and the interaction between them as independent factors, and the number of offspring per female and the offspring sex ratio, as dependent variables.

To test the effect of *Wolbachia* on the female reproductive success at the individual level, we performed generalized linear mixed models, with the treatment ("W^+" or "W^-"), female physiological age (5 or 10 days), and the interaction between them as fixed factors, and the group of fleas with which they were daily fed together on a host, as a random factor. We conducted one analysis with the integrated reproductive success index as a dependent variable. We then added separate analyses to investigate the relative importance of different fitness components by considering the offspring development time from egg to emerging adult, the number of offspring per female, their body size, the sex ratio (proportion of females), and the probability of offspring survival under starvation, each separately as dependent variables.

To account for sexual polymorphism in development time and body size, male and female offspring were analyzed separately in the relevant analyses. To account for sexual polymorphism in the survival time of offspring

(Krasnov, 2008), we standardized these values. This was done by converting the survival time of each offspring (e.g., a female that survived 18 days) to the probability of fleas from its own sex to survive until this day (e.g., probability of female fleas to survive 18 days), using the packages "survival" and "survminer" (R Core Team, 2020). For the analysis of the sex ratio that may largely depend on the total number of offspring per female, we added the offspring number as a covariant. All analyses were performed by using the GLM and GLMM packages lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017; R Core Team, 2020).

RESULTS

The tetracycline supplementation worked efficiently, as even after three antibiotic-free generations, there was no indication of *Wolbachia* in any of the treated fleas that were tested (N = 88 parental females, N = 13 female offspring, and N = 20 parental males). In contrast, as expected, all female fleas from the control group (N = 79 parental female and N = 13 female offspring) and six of the 20 parental control males were *Wolbachia*-positive. Consistent with the *Wolbachia* loads observed in wild fleas (Flatau et al., 2018), loads were significantly higher for control females than for control males ($3 \times 10^5 \pm 2 \times 10^5$, for females, and $7 \times 10^3 \pm 1 \times 10^4$, for males).

Wolbachia presence in control female fleas and absence in female fleas of the *Wolbachia*-negative treatment were further confirmed by the FISH analyses (**Figure 2**). In control females, loads of *Wolbachia* cells were detected in the ovaries and Malpighian tubules, but not in the saliva gland, midgut, or spermatheca (**Figure 2**). No *Wolbachia* cells were detected in any of the male organs of either treatment groups. This failure to detect *Wolbachia* in any of the males including those that were not treated by antibiotics was possibly because *Wolbachia* titers were too low to be detected by the FISH protocol that we used (Schneider et al., 2018).

At the group level, we only found an age effect on the number of offspring per female, where the number of offspring increased with the parent female's physiological age. Neither treatment, age, nor the interaction between the two had a significant effect on the offspring sex ratio (**Table 1**).

Similarly, at the individual level, the physiological age of the female parent significantly affected the integrated index of reproductive success, the number of offspring per female, and offspring developmental time (**Table 1**). In contrast to our predictions, there was also no effect of the treatment or treatment × age interaction on any of the dependent variables at this level.

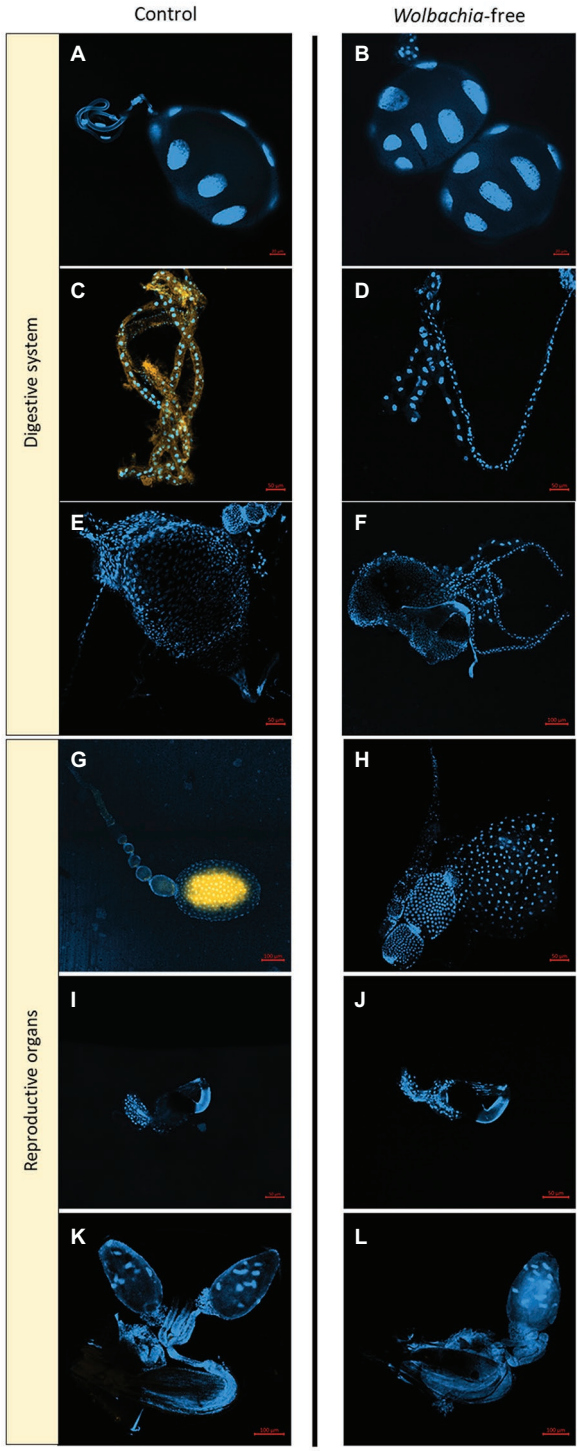

FIGURE 2 | *Wolbachia* localization within *Synosternus cleopatrae* fleas. Fluorescence *in situ* hybridizations of organs in the ingestion and reproductive systems of control (W^+, left) and *Wolbachia*-free (W^-, right) fleas. **(A,B)** saliva gland, **(C,D)** Malpighian tubules, **(E,F)** midgut, **(G,H)** ovary, **(I,J)** spermatheca, and **(K,L)** male genitalia. DAPI stain for double-stranded DNA is indicated in blue and the specific probe for *Wolbachia* is indicated in yellow. For optimal illustration, except male genitalia, all other organs were taken from female fleas, although male fleas were also tested (see text).

DISCUSSION

Three main mechanisms are commonly proposed to explain the temporal and spatial persistence of arthropod endosymbionts in their host populations: **obligatory relationships**, in which

TABLE 1 | Experimental results.

Group-level analysis			
Dependent variable	**W⁺**	**W⁻**	**F statistics**
Mean offspring number per female	$Y = -0.3 + 0.4X$ $R^2 = 0.8$	$Y = -0.3 + 0.5X$ $R^2 = 0.8$	Age: 308*** Treatment: 1 Age × Treatment: 0.62
Sex ratio	$Y = 0.6 - 0.02X$ $R^2 = 0.04$	$Y = 0.5 - 0.002X$ $R^2 = 0.0007$	Age: 0.8 Treatment: 0.04 Age × Treatment: 0.4

Individual-level analysis					
Dependent variable	**W⁺ d 5**	**W⁻ d 5**	**W⁺ d 10**	**W⁻ d 10**	**T statistics**
Reproductive success index	13 ± 3	11 ± 3	20 ± 3	20 ± 3	Age: 3* Treatment: 0.7 Age × Treatment: 0.5
Offspring number per female	2 ± 0.4	2 ± 0.4	3 ± 0.4	4 ± 0.4	Age: 2.8* Treatment: 0.9 Age × Treatment: −0.9
Sex ratio	0.5 ± 0.05	0.5 ± 0.05	0.6 ± 0.05	0.5 ± 0.05	Age: −0.4 Treatment: 0.3 Age × Treatment: 0.6
Offspring survival	0.5 ± 0.04	0.6 ± 0.05	0.5 ± 0.04	0.5 ± 0.05	Age: −1 Treatment: −0.9 Age × Treatment: 0.8
Offspring male development	41 ± 0.3	42 ± 0.3	40 ± 0.3	40 ± 0.3	Age: −5*** Treatment: −2 Age × Treatment: 2
Offspring female development	34 ± 0.2	35 ± 0.3	32 ± 0.2	32 ± 0.2	Age: −4** Treatment: −2 Age × Treatment: 1
Offspring male body size	7 ± 0.1	7 ± 0.3	7 ± 0.1	7 ± 0.1	Age: 0.2 Treatment: 0.1 Age × Treatment: −0.2
Offspring female body size	13 ± 0.1	13 ± 0.1	14 ± 0.1	13 ± 0.1	Age: 1 Treatment: −0.1 Age × Treatment: 0.3

*Means ± standard errors of the fitness-correlated parameters assessed for Wolbachia-free (W⁻) and control (W⁺) female fleas at various ages (day 5 and day 10 correspond to age 5 days and age 10 days, respectively) by the group and individual level analyses. Statistical results are provided on the right side. *p < 0.05; **p < 0.01; ***p < 0.001.*

the host is fully dependent on its endosymbiont, **fitness advantages** conferred by the endosymbiont, and **reproductive manipulations** imposed by the endosymbiont. To shed light on the universality of these mechanisms across host-endosymbiont systems, we experimentally explored the interactions between *Wolbachia* and their flea host, which at the first glance, seem to differ from those in most documented systems, due to the extremely high persistence levels over time and space in female, but not in male, hosts. Our results do not support any of the tested mechanisms, as neither fitness estimates nor sex ratio differed between control fleas and fleas cured of *Wolbachia*. Below, we discuss these results and highlight future directions required to better understand the interactions between *S. cleopatrae* fleas and their *Wolbachia* endosymbionts, as well as other systems with extreme sex-differences in endosymbiont persistence.

Hypothesis 1 Is Not Supported: The Interaction With the Endosymbiont Is Not Obligatory for Either Female or Male Hosts

Our results refute this hypothesis as both female and male fleas were able to survive and reproduce for multiple generations without the presence of *Wolbachia*, confirming that the interaction is not obligatory for either sex. Hence, *Wolbachia* should be considered as a facultative endosymbiont in this system, and thus, its potential effects on the host can range from negative (Vorburger and Gouskov, 2011), through neutral (Moran et al., 2008), to positive (Brownlie et al., 2009; Vorburger and Gouskov, 2011).

Likewise, other model systems with potential sex-differences in endosymbiont persistence (as suggested by sex-differences in endosymbiont prevalence) seem to have facultative relationships with both female and male hosts. This is reflected by the occurrence of some female host individuals that did not carry

the endosymbiont (e.g., *Rickettsia-Scymnus frontalis*, *Spiroplasma-Adalia punctate*, *Spiroplasma-Anisosticta punctate*, *Wolbachia-Coccidula rufa*, *Wolbachia-Rhyzobius litura*, *Wolbachia-Sogatella furcifera*, *Wolbachia-Ctenocephalides canis*, and "*Candidatus* Midichloria mitochondrii"-*Ixodes ricinus*; Noda et al., 2001; Gorham et al., 2003; Weinert et al., 2007; Sassera et al., 2008) or by the successful survival and reproduction of endosymbiont-cured hosts (e.g., *Wolbachia-Drosophila pseudotakahashii*, Richardson et al., 2019). Long-term field surveys are required to confirm whether the above-mentioned model systems indeed demonstrate sex-differences in endosymbiont persistence over time and space. If they do, these studies, taken together, may suggest that such a persistence pattern is not necessarily related to the obligatory dependence of the females on the endosymbiont.

Hypothesis 2 Is Not Supported: There Is No Evidence for Fitness Advantages for Female Hosts Carrying the Endosymbiont

We found no support for this hypothesis, as there was no reduction in the reproductive success of females cured of *Wolbachia*, either when they were tested individually or in a group (**Table 1**). Investigations at the individual level allowed us to obtain a high-resolution snapshot view of *Wolbachia* effects on the current reproductive success of female fleas at early (5 days) and older ages (10 days), which are typically associated with low and high *Wolbachia* loads, respectively (Flatau et al., 2018). The group level complemented the individual level analysis by providing continuous information on daily reproduction in a more realistic social environment, including multiple female and male fleas.

Similar to our study, the only other two model systems with potential sex-differences in endosymbiont persistence, in which fitness effects were tested, failed to demonstrate differences between the reproductive success of control and endosymbiont-cured hosts (*Wolbachia* and *Sogatella furcifera* or *Drosophila pseudotakahashii*; Noda et al., 2001; Richardson et al., 2019). This may suggest that the high population persistence of endoparasites in female hosts may be maintained without obvious fitness advantages for the host.

The exploration of endosymbiont effects on its host fitness is almost a standard practice in symbiosis studies. This can be done either by relying on the natural variation in endosymbiont load while correlating it with host-fitness-related traits (e.g., Unckless et al., 2009; Segoli et al., 2013) or by directly manipulating endosymbiont presence *via* curing or infecting the host (Koga et al., 2007; Da et al., 2016; Karimi et al., 2019). Our results emphasize the limitations of the correlative approach, as when we previously adopted such an approach, we found potential evidence for *Wolbachia*-negative effects on *S. cleopatrae* females (Flatau et al., 2018). Such negative effects were not detected *via* experimental manipulation in the current study and, instead, were likely the result of a confounding effect between the flea age and *Wolbachia* load. Thus, we encourage researchers to face the challenges entailed by endosymbiont curing, endosymbiont injection, or both, to better characterize host-endosymbiont relationships.

Notably, our experiments were conducted under favorable laboratory conditions. To complement the view of *Wolbachia* fitness effects on its host, future experiments should be conducted under more demanding conditions, e.g., food limitation, high competition, and enemy presence (Brownlie et al., 2009; Gavotte et al., 2010; Vorburger and Gouskov, 2011), and on earlier developmental stages (Da et al., 2016). For example, Cao et al. (2019) found that *Wolbachia* provides fitness advantages to its *Drosophila simulans* host only when breeding on fungi-infected fruits. In another example, Brownlie et al. (2009) showed that *Wolbachia* infection confers a positive fecundity benefit for *D. melanogaster* only when they were reared on iron-restricted or overloaded diets.

In particular, several lines of evidence suggest potential fitness advantages that may be more pronounced in *S. cleopatrae* females than in male fleas under unfavorable conditions. First, although we have not quantified *Wolbachia* in flea eggs and larvae, the FISH analysis of control females suggests that all eggs carry *Wolbachia*. This implies that males may experience a secondary loss of infection during their development, which could be indicative of a facultative nutritional role of *Wolbachia*, since females have greater nutritional needs than males in blood sucking arthropods (e.g., Krasnov, 2008; Ben-Yosef et al., 2020). Second, the dominant occurrence of *Wolbachia* in the Malpighian tubules of *S. cleopatrae* females (**Figure 2**) may further support a nutritional role (Ben-Yosef et al., 2020; Reis et al., 2020) or indicate that these organs may store *Wolbachia* for other beneficial functions (Faria and Sucena, 2013). Altogether, this evidence may suggest that under restricted conditions, e.g., low nutrient availability for the larvae, anemic hosts for adult fleas, or exposure to pathogens, some potential *Wolbachia* fitness advantages to females may be expressed.

Interactions with other bacteria could also play a role. In particular, coinfection by *Bartonella* spp., the second most abundant bacteria in wild *S. cleopatrae* (Cohen et al., 2015), which is absent in the laboratory colony of fleas, may lead to *Wolbachia* fitness advantages on their flea hosts, if high *Bartonella* loads damage the fleas. Indeed, Morick et al. (2013) found an indication of fitness reduction in *Xenopsylla ramesi* fleas in the presence of *Bartonella*. Therefore, growing the flea under limited nutritional or high competition conditions or in the presence of other bacteria could potentially reveal "hidden" fitness advantages in this system, as well as in other systems with sex-differences in endosymbiont persistence.

Hypothesis 3 Is Not Supported: There Is No Indication for Reproductive Manipulation Inducing Female-Biased Sex Ratio

Wolbachia presence had no effect on the offspring sex ratio, ruling out the possibility of sex-distorting manipulations, such as MK, IP, and MF, which induce female-biased sex ratio in their host (Werren et al., 2008). The fourth common reproductive manipulation, CI, seems unlikely as well, owing to the absence or near absence of *Wolbachia* in males (Bourtzis et al., 1996; Werren et al., 2008; Richardson et al., 2019). However, since

CI is not predicted to cause female-biased sex ratio in arthropods (Werren et al., 2008), we cannot rule out this possibility. Moreover, recent evidence suggests that *Wolbachia* can cause CI or at least partial CI, despite its low densities in males (Noda et al., 2001; Richardson et al., 2019). Interestingly, the evidence for a full CI by male fleas with no detected *Wolbachia* comes from a *Wolbachia-Drosophila* model system that exhibited an extreme female-biased *Wolbachia* persistence pattern as in our study system, with 100% prevalence and high *Wolbachia* loads in females (cycle number at detection threshold, Cp 27.4 ± 2.7), and low prevalence (36%) and loads in males (Cp 35.3 ± 2.7). Unfortunately, apart from the two above-mentioned model systems, which show indications for CI (Noda et al., 2001; Richardson et al., 2019), to the best of our knowledge, the possibility of reproductive manipulations has not been explored in the other systems with potential sex-differences in endosymbiont persistence.

Proposed Underlying Mechanisms for the Persistence of Endosymbionts With Sex-Biased Infection Patterns

Our findings, combined with that of others (Noda et al., 2001; Gorham et al., 2003; Weinert et al., 2007; Richardson et al., 2019), highlight the question: how do endosymbionts persist in systems with extreme sex-biased endosymbiont infection levels? As noted above, CI and fitness advantages for female hosts, under limiting conditions, are two plausible mechanisms that should be further investigated.

An alternative possibility is that past reproductive manipulation or fitness effects allowed the endosymbiont to spread in the host population initially, and the high persistence in females is currently maintained *via* high transmission rates. For example, evidence suggests that in the butterfly *Hypolimnas bolina*, male killing has occurred in the past but no longer operates, while *Wolbachia* still persist at high prevalence in host populations (Charlat et al., 2005). Inspired by the "ghost of competition past" (Connell, 1980), we term this alternative hypothesis as the "ghost of past manipulations or fitness effects." This hypothesis is supported by theoretical models, showing that a high maternal transmission rate can ensure endosymbiont persistence in the population in the absence of a reproductive manipulation (Prout, 1994; Turelli, 1994; Richardson et al., 2019). Moreover, an evolutionary increase in the transmission rate, at the expense of reproductive manipulation, is consistent with the low endosymbiont prevalence in males. This is because selection to maintain high transmission to males may not be as strong as selection to maintain high transmission to females, considering that males constitute an evolutionary dead-end for the symbiont (Normark, 2004).

In conclusion, we found no evidence for any of the suggested underlying mechanisms for the high endosymbiont persistence in female fleas, calling for additional exploration of hidden fitness effects, the occurrence of CI, or alternative mechanisms, in this particular system, as well as in additional systems with sex-bias differences in endosymbiont persistence. A better understanding of the persistence mechanisms in a variety of systems will shed further light on the evolution and ecology of arthropod-endosymbiont interactions in nature.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The handling protocol was approved by the committee for the ethical care and use of animals in experiments of Ben-Gurion university of the Negev (IL-85-10-2019b).

AUTHOR CONTRIBUTIONS

All authors contributed to the concept and design of the study. RF established the cured and infected line and conducted the feeding and reproduction experiment and all the molecular analyses. RF and HH performed the statistical analysis. All authors contributed to the manuscript preparation and read and approved the submitted version.

FUNDING

This study was supported by the Israel Science Foundation (ISF), grant number 1391/15 to HH.

ACKNOWLEDGMENTS

We thank M. Ghanim, A. Hoffmann, I. Khokhlova, N. Knossow, B. Krasnov, R. Rodríguez Pastor, T. Rozenberg and N. Sikron for valuable advice and technical support during this study.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 April 2021
doi: 10.3389/fmicb.2021.642543

Patterns of Microbiome Variation Among Infrapopulations of Permanent Bloodsucking Parasites

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While interspecific variation in microbiome composition can often be readily explained by factors such as host species identity, there is still limited knowledge of how microbiomes vary at scales lower than the species level (e.g., between individuals or populations). Here, we evaluated variation in microbiome composition of individual parasites among infrapopulations (i.e., populations of parasites of the same species living on a single host individual). To address this question, we used genome-resolved and shotgun metagenomic data of 17 infrapopulations (balanced design) of the permanent, bloodsucking seal louse *Echinophthirius horridus* sampled from individual Saimaa ringed seals *Pusa hispida saimensis*. Both genome-resolved and read-based metagenomic classification approaches consistently show that parasite infrapopulation identity is a significant factor that explains both qualitative and quantitative patterns of microbiome variation at the intraspecific level. This study contributes to the general understanding of the factors driving patterns of intraspecific variation in microbiome composition, especially of bloodsucking parasites, and has implications for understanding how well-known processes occurring at higher taxonomic levels, such as phylosymbiosis, might arise in these systems.

Keywords: genome-resolved metagenomics, host-symbiont, intraspecific variation, lice, microbiota, shotgun metagenomics, symbiont

OPEN ACCESS

Edited by:
Hirokazu Toju, Kyoto University, Japan

Reviewed by:
Martin Stoffel, University of Edinburgh, United Kingdom
Hideyuki Doi, University of Hyogo, Japan

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Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *16 December 2020*
Accepted: *26 March 2021*
Published: *16 April 2021*

Citation:
Doña J, Virrueta Herrera S, Nyman T, Kunnasranta M and Johnson KP (2021) Patterns of Microbiome Variation Among Infrapopulations of Permanent Bloodsucking Parasites. Front. Microbiol. 12:642543. doi: 10.3389/fmicb.2021.642543

INTRODUCTION

Patterns of inter- and intraspecific variation in microbiome composition of animals have received much attention because the microbiome may influence many biological processes that have considerable effects on the host (Clemente et al., 2012; Le Chatelier et al., 2013; Rothschild et al., 2018; Rudman et al., 2019; Velazquez et al., 2019). For instance, particular microbiome compositions have been found to drive genomic adaptation (Rudman et al., 2019) or to confer protection against pathogens (Velazquez et al., 2019).

In general, both stochastic (e.g., dispersal, or ecological drift) and deterministic (e.g., host immunological regulation, or microbe–microbe interactions) processes operate across multiple spatial scales to shape the composition of animal microbiomes (Adair and Douglas, 2017;

Kohl, 2020). In particular, among the many determinants shaping microbiome composition, host species identity has been repeatedly identified as a key factor determining the composition of animal microbiomes (Brooks et al., 2016; Mazel et al., 2018; Nishida and Ochman, 2018; Knowles et al., 2019; Lutz et al., 2019; Lim and Bordenstein, 2020; Song et al., 2020). In other words, microbiomes of individuals of the same species tend to be more similar than to those of another species. This pattern is generally the result of filtering microbial taxa by the host (e.g., through host diet, habitat, or immune system, Adair and Douglas, 2017) or result from host–microbe coevolution (Lim and Bordenstein, 2020). When this process exhibits phylogenetic signal, the pattern is known as phylosymbiosis (i.e., microbial community relationships that recapitulate the phylogeny of their host, Brucker and Bordenstein, 2013; Brooks et al., 2016; Lim and Bordenstein, 2020). Nonetheless, several aspects of the variation of animal microbiomes are yet to be better understood (Lim and Bordenstein, 2020). In particular, for non-human animals, there is still much to learn about how microbiomes vary at scales below the species level, such as between populations (Blekhman et al., 2015; Kohl et al., 2018; Rothschild et al., 2018; Campbell et al., 2020; Fountain-Jones et al., 2020) or ecotypes (Agany et al., 2020).

An area of focus on understanding intraspecific variation in microbiome composition has been bloodsucking parasites. In these parasites, previous studies have consistently found a major role of the host species in shaping microbiome composition in the parasites (Osei-Poku et al., 2012; Zhang et al., 2014; Swei and Kwan, 2017; Zolnik et al., 2018; Díaz-Sánchez et al., 2019; Landesman et al., 2019; Lee et al., 2019; Muturi et al., 2019). However, in ticks (*Ixodes scapularis*), host individual identity of the blood meal was even more important than host species identity in explaining microbiome composition (Landesman et al., 2019). These results suggested that individual host identity of the blood meal might be an important factor that shapes parasite microbiomes at the intraspecific level (Landesman et al., 2019). In theory, microbiomes of individual bloodsucking parasites could vary due to: (1) the individual parasite immune system that may impose selection on different bacterial taxa (Blekhman et al., 2015; Suzuki et al., 2019); (2) differences in the source of the blood meal that may transfer or disperse particular bacterial taxa, or modulate bacteria by creating specific conditions during digestion (Rothschild et al., 2018); (3) microbe–microbe interactions (Hassani et al., 2018); and (4) stochastic processes (e.g., ecological drift) (Lankau et al., 2012). However, for most species, and for bloodsucking parasites in particular, the nature of intraspecific variation in microbiomes and the relative importance of factors shaping this variation remain understudied.

Sucking lice (Phthiraptera: Anoplura) are permanent blood-feeding ectoparasites that live in the fur or hairs of mammals. Anopluran lice have been shown to host intracellular bacterial endosymbionts that are likely to help to complement deficiencies in their diet, and these symbionts tend to be located on specialized structures known as mycetomes (Buchner, 1965; Boyd and Reed, 2012; Sasaki-Fukatsu et al., 2006; Perotti et al., 2007, 2008). Previous studies have found that members of Anoplura host a single endosymbiont, but belonging to different bacterial genera depending on louse species, including *Riesia* (Sasaki-Fukatsu et al., 2006; Kirkness et al., 2010; Boyd et al., 2014), *Sodalis* (Boyd et al., 2016), and *Legionella* (Říhová et al., 2017). We also know from these studies that, as in other arthropod endosymbionts, louse endosymbionts tend to have reduced genomes (Kirkness et al., 2010; Boyd et al., 2017). On the other hand, processes such as replacement (i.e., the substitution of one endosymbiotic species by another) and independent acquisitions of different endosymbionts can occur across evolutionary time scales (i.e., millions of years) (Sasaki-Fukatsu et al., 2006; Allen et al., 2007, 2016; Hypša and Køížek, 2007; Fukatsu et al., 2009). Thus, while there is some background knowledge on louse endosymbionts, several aspects are yet to be understood. For instance, how microbiomes vary across organs, systems, or individuals of lice from the same species is mostly unknown, with a single study to date providing bacterial community data for different individual lice of the same species (Říhová et al., 2019).

The sucking lice of pinnipeds (seals, sea lions, and walrus) are of particular interest because of their need to adapt to the aquatic lifestyle of their hosts (Durden and Musser, 1994; Leonardi et al., 2013). There is evidence that the sucking lice of seals and sea lions have codiversified with their hosts (Kim, 1971, 1975, 1985; Leonardi et al., 2019). Indeed, the sucking lice of pinnipeds represent an interesting system in which to study the variation in microbiome composition and the drivers of this variation at an intraspecific level because: (1) these lice have well defined, isolated populations (infrapopulations) on individual seal hosts, due to an expected low rate of horizontal dispersal among host individuals, which is only possible during the seals' haul-out periods on land or ice (Kim, 1985; Leonardi et al., 2013, 2019); and (2) these lice feed only upon the blood of their host (Snodgrass, 1944; Kim, 1985), so that it can be assumed that individuals from the same infrapopulation feed upon "exactly" the same resource (i.e., the blood of the individual seal on which they occur). In addition, previous studies conducted on seal microbiomes have found that while factors such as species identity, age, sex, and diet play a role in shaping seal microbial communities, seals show evidence of a core microbiome with which they have co-evolved (Nelson et al., 2013; Acquarone et al., 2020; Kim et al., 2020; Stoffel et al., 2020).

Here, we used genome-resolved approaches (the construction of draft microbial genomes from short-read shotgun sequencing data; Bowers et al., 2017; Uritskiy et al., 2018) and metagenomic classification tools (taxonomic classification of individual sequencing reads; Menzel et al., 2016) to infer patterns of microbiome variation among individuals of the sucking seal louse *Echinophthirius horridus* (von Olfers, 1816) inhabiting individual Saimaa ringed seals *Pusa hispida saimensis* (Nordquist, 1899). These two approaches have different limitations and strengths. For example, the genome-resolved approach allows the assembly of multiple highly complete bacterial genomes, but only for organisms with enough coverage to be assembled and binned. On the other hand, metagenomic classification of reads may offer a more comprehensive picture of community composition because of higher database completeness or less strict thresholds to analyze data. However, read classification

is limited by the fact that it is based only on the fraction of reads that map to reference databases (Quince et al., 2017). Our sampling design, involving analysis of two individual lice from each of 17 seals, allowed us to evaluate the degree to which variation in microbiome composition among individual lice is explained by the infrapopulation (the identity of the seal host).

MATERIALS AND METHODS

Sampling, DNA Extraction, and Sequencing

Thirty-four individual lice were sampled from 17 individual Saimaa ringed seals (*Pusa hispida saimensis*), which is an endemic endangered landlocked subspecies of the ringed seal living in freshwater Lake Saimaa in Finland (e.g., Nyman et al., 2014). Individual lice were collected from seals found dead or from seals that were live-captured for telemetry studies (e.g., Niemi et al., 2019), and placed in 2-ml screw-cap tubes with 99.5% ethanol. Lice from a single seal individual were put in the same tube. Prior to DNA extraction, each louse individual was rinsed with 95% ethanol and placed alone in a new sterile vial; then, the remaining ethanol was evaporated at room temperature.

Whole lice were ground up individually, and genomic DNA was extracted using the Qiagen QIAamp DNA Micro Kit (Qiagen, Valencia, CA, United States). The standard protocol was modified so that specimens were incubated in ATL buffer and proteinase K at 55 (insert degree) C for 48 h instead of the recommended 1–3 h, as well as by substituting buffer AE with buffer EB (elution buffer). This was done to ensure maximal yield (greater than 5 ng) of DNA from each louse. Each DNA extract was quantified with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, United States) following the manufacturer's recommended protocols.

Shotgun genomic libraries were prepared from the extracts with Hyper Library construction kits (Kapa Biosystems, Wilmington, MA, United States). The libraries were quantitated by quantitative PCR (qPCR) on a Bio-Rad CFX Connect Real-Time System (Bio-Rad Laboratories, Inc., CA, United States) and 150 bp pair-end sequenced on either one of two lanes (**Supplementary Table 1**) of an Illumina NovaSeq 6000 sequencer (Albany, New York, United States). FASTQ files from sequence data were generated and demultiplexed with bcl2fastq v.2.20. All library preparations, sequencing, and FASTQ file generation were carried out at the Roy J. Carver Biotechnology Center (University of Illinois, Urbana, IL, United States). Raw reads were subsequently deposited to the NCBI GenBank SRA database (**Supplementary Table 1**).

Metagenomic Analyses

For the genome-resolved metagenomic analyses, we used the metaWRAP v1.1.5 pipeline (Uritskiy et al., 2018) along with all the recommended databases (i.e., Checkm_DB, NCBI_nt, and NCBI_tax). We used the metaWRAP Read_qc module with default parameters to quality trim the reads and to de-contaminate each sample from host reads. For decontamination, we ran a *de novo* genome assembly of an individual louse of the same species, not included in this study, and with a high amount of sequencing data ("Echor52") in Abyss v2.0.1 (Jackman et al., 2017). Finally, we filtered out all non-bacterial reads from the contig file using Blobtools v1.0.1 (Laetsch and Blaxter, 2017) and used this file to decontaminate all the other samples with the metaWRAP Read_qc module. See **Supplementary Table 1** and Data Availability section for more details on the data preprocessing results. Next, we co-assembled reads from all the samples with the metaWRAP Assembly module (–use metaspades option) (Nurk et al., 2017). For this assembly, and because of memory limitations, we used BBNorm[1] before assembly to reduce the coverage of the concatenated FASTQ file to a maximum of 100× and to discard reads with coverage under 3×. We binned reads with the metaWRAP Binning module (–maxbin2 –concoct –metabat2 options) (Alneberg et al., 2014; Wu et al., 2016; Kang et al., 2019) and then consolidated the resulting bins into a final bin set with both metaWRAP's Bin_refinement module (-c 50 -× 10 options) and the Reassemble_bins module. We quantified the bins resulting from the Bin_refinement module with Salmon (Patro et al., 2017) using the Quant_bins module with default parameters. Finally, we classified bins using the Classify_bins module. This module uses Taxator-tk, which gives highly accurate but conservative classifications (Dröge et al., 2015). Accordingly, we also uploaded our final metagenome-assembled genomes (MAGs) to MiGA for a complementary analysis to determine the most likely taxonomic classification and novelty rank of the bin (Rodriguez-R et al., 2018). We used the NCBI Genome database (Prokaryotes; February 26, 2020 version) for this analysis.

For the metagenomic classification of reads, we used the metagenomic classifier Kaiju (Menzel et al., 2016) with Reference database: nr (Bacteria and Archaea; Database date: 2017-05-16). We used the default parameters for these analyses (SEG low complexity filter: yes; Run mode: greedy; Minimum match length: 11; Minimum match score: 75; Allowed mismatches: 5). We then converted Kaiju's output files into a summary table at the genus and species level and filtered out taxa with low abundances (<0.1% of the total reads). We also removed poorly identified taxa because they would artificially increase the similarity between our samples. Specifically, the following taxa were excluded: "NA," "Viruses," "archaeon," "uncultured bacterium," "uncultured Gammaproteobacteria bacterium" (**Supplementary Tables 2, 3**).

Lastly, we used Decontam v1.2.1 to filter out bacterial taxa exhibiting known statistical properties of contaminants (Davis et al., 2018). We used the frequency method (*isContaminant* function) which is based on the inverse relationship between the relative abundance of contaminants and sample DNA concentration, and also has been found suitable for samples dominated by host DNA (Willner et al., 2012; Lusk, 2014; Salter et al., 2014; Jervis-Bardy et al., 2015; Hooper et al., 2019; McArdle and Kaforou, 2020). As input for Decontam analyses, we used the aforementioned total DNA concentration values. Then, as recommended, we explored the distribution of scores assigned by Decontam to assign the threshold according to bimodality between very low and high scores (Davis et al., 2018). For the

[1]https://sourceforge.net/projects/bbmap/

MAGs matrix, no bimodality was found, and thus we used the 0.1 default value (**Supplementary Figure 1A**). None of the MAGs were classified as contaminants, according to Decontam. For Kaiju matrices, a 0.3 threshold value was selected for the species-level matrix (**Supplementary Figure 1B**) and 0.31 for the genus-level matrix (**Supplementary Figure 1C**). Decontam filtered out a single species (*Clostridia* bacterium k32) from the species matrix and two genera (*Cupriavidus* and *Massilia*) from the genus matrix.

Statistical Analyses

For the genome-resolved metagenomic analyses, we used the normalized MAGs compositional matrices resulting from Salmon. Specifically, these MAG counts are standardized to the individual sample size (MAG copies per million reads) and thus allow between-sample comparisons. For the Kaiju analyses, we used the rarefy_even_depth function of phyloseq (without replacement as in the hypergeometric model) to rarefy samples to the smallest number of classified sequences per individual observed (85,513 and 71,267 reads in genus and species matrices, respectively) (Weiss et al., 2017).

To visualize similarities of microbiome composition among louse individuals from the same or different individual seal hosts, we constructed non-metric multidimensional scaling (NMDS) ordinations based on Bray–Curtis and Jaccard (binary = T) dissimilarities using the phyloseq v1.26-1 R package (McMurdie and Holmes, 2013). Also, to remove subjective bias when interpreting the results of our main NMDS ordinations, we also analyzed whether Bray–Curtis dissimilarity distances between samples from the same infrapopulation were lower than those of comparisons with samples from other infrapopulations. We statistically tested these results using two-sided Sign tests, with which we evaluated whether the difference between within- versus among-infrapopulation medians was significantly different from 0.

To assess the influence of individual host identity on the microbiome composition of louse individuals, we conducted a permutational multivariate analysis of variance (PERMANOVA) (Anderson and Walsh, 2013; Anderson, 2014). PERMANOVA analyses were done using the *adonis2* function in vegan v2.5–4 (Oksanen et al., 2019), based on Bray–Curtis and Jaccard distance matrices with 100 iterations. In PERMANOVA analyses, for the individual host identity factor, our within-group sample size (n = 2) was smaller than both the total number of groups (n = 17) and the total sample size (n = 34). From the perspective of statistical power for testing effects related to host individuals, the relatively high number of hosts, in essence, offsets the low number of lice per host. Nevertheless, to account for a potential deviation in F-statistics and R^2 values (Kelly et al., 2015), we wrote an R simulation that randomly subsampled the infrapopulations from which the louse came (5 infrapopulations per iteration). We ran 10 iterations and ran a PERMANOVA analysis for each iteration. Note that, for a few iterations, subsampled samples were too similar and PERMANOVA could not be done. In addition, we ran PERMANOVA analyses to explore additional factors (louse sex: male, female; sequencing lane: 1, 2; and host status: dead, alive) that may explain variance in microbiome composition. Furthermore, we included significant factors as the first factors of the host identity PERMANOVA models (i.e., to obtain the variance explained by host identity after accounting for the variance explained by that factor). We also restricted the groups in which permutations could be done to only those with the same value of that vector using the strata argument (e.g., for a sample collected from a dead host, and for the host-status factor, permutations could only be done among other dead hosts). Lastly, we ran a Mantel test using the *mantel* function in vegan (method = spearman, permutations = 9999) to explore if host locality (i.e., the coordinates in which each host was sampled) correlated with the microbiome composition of louse individuals. For this analysis, we ran 10 iterations of an R simulation in which we randomly subsampled one louse sample for each individual host and ran a Mantel test for each iteration. The following packages were used to produce the plots: ggplot2 v3.1.0.9 (Wickham, 2016), grid v3.5.3 (R Core Team, 2019), gridExtra v.2.3 (Auguie, 2016), ggrepel v0.8.0 (Slowikowski et al., 2019), ggpubr v.0.2.5 (Kassambara, 2018), and ggsci v2.9 (Xiao, 2018).

RESULTS

From the genome-resolved metagenomics pipeline, 13 high-quality bacterial metagenome-assembled genomes (MAGs) were obtained (**Table 1** and **Figure 1**). According to MiGA analyses, 10 of them (77%) likely belong to a species not represented in the NCBI Genome database.

Kaiju analyses recovered a higher diversity of microorganisms than did the genome-resolved approach (**Figure 2** and **Supplementary Figure 2**). These differences are likely because of the quality-filtering parameters used in the genome-resolved metagenomics pipeline (i.e., these taxa may have been discarded because the completeness values of their bins were lower than 50% and/or their contamination values were higher than 10%). Nevertheless, bacterial taxa found in the genome-resolved metagenomic approach were generally found also in Kaiju and with similar relative abundances (**Figure 2**), and a similar pattern was found also at the species level (**Supplementary Figure 2**).

Non-metric multidimensional scaling ordinations and PERMANOVA analyses show a major role of infrapopulation identity in explaining microbiome composition for both quantitative and presence–absence data. In the genome-resolved metagenomic dissimilarity matrices, the Bray–Curtis–based NMDS ordination evidenced a strong pattern of clustering by infrapopulation identity (**Figure 3A**). This pattern was not noticeable in the Jaccard-based NMDS ordination because dissimilarity was too low among samples (**Supplementary Figure 3A**). PERMANOVA analyses indicated that most (>84% in all cases) of the variance was explained by infrapopulation identity (PERMANOVA: Bray–Curtis, R^2 = 0.857, F = 6.419, P = 0.001, **Figure 3A**; Jaccard, R^2 = 0.842, F = 5.671, P = 0.001; **Supplementary Figure 3A**). The analyses of differences in pairwise distances showed highly consistent results, as pairwise distances between samples from the same infrapopulations were lower than the median of among-infrapopulation pairwise comparisons in 16 out of 17 infrapopulations (94%; Sign test:

TABLE 1 | Statistics of the MAGs assembled.

MAG name	Completeness (%)	Contamination (%)	N50 (bp)	Size (bp)	Taxator tk ID	MiGA ID	RDP ID	Taxonomic novelty
bin.1	100	1.07	57370	1869975	Flavobacteriaceae	Flavobacteriaceae*	NA	Species****
bin.4	99.26	0.24	81315	2500734	Flavobacteriaceae	Chryseobacterium*	Chryseobacterium (100.0%)	Species****
bin.2	98.51	0.42	36844	3101576	Deinococcus	Deinococcus grandis*	Deinococcus (100.0%)	Subspecies****
bin.7	97.75	0	16123	2650064	Moraxellaceae	Psychrobacter sp. PRwf-1*	NA	Subspecies****
bin.3	97.41	1.33	32961	4014303	Neisseriales	Pseudogulbenkiania*	NA	Species****
bin.11	95.65	0.92	69243	2786419	Moraxellaceae	Psychrobacter*	NA	Species****
bin.10	95.12	0	13409	2459723	Deinococcaceae	Deinococcus*	NA	Species****
bin.12	93.14	0.85	24793	2851493	Deinococcaceae	Deinococcus*	NA	Species****
bin.6	88.74	1.45	7283	1988194	Micrococcales	Arthrobacter*	NA	Species****
bin.13	77.11	0.64	3045	2627969	Deinococcaceae	Deinococcus*	NA	Species****
bin.5	74.13	0.61	24837	1635952	Moraxellaceae	unclassified Moraxellaceae*	Alkanindiges (99%)	Species****
bin.8	67.76	0	10934	2837743	Deinococcaceae	Deinococcus*	NA	Species****
bin.9	61.13	0.30	2210	2110411	Janthinobacterium	Janthinobacterium sp. SNU WT3***	NA	Subspecies****

*MAG name indicates the name given to that bin for this study (e.g., in **Figure 1**). The highest taxonomic rank with p-value ≤ 0.5 is shown in MiGA ID. RPD ID is the result of the identification analysis using rRNA genes (16S) implemented in MiGA; % indicates confidence in identification. Taxonomic novelty is a MiGA analysis that indicates the taxonomic rank at which the MAG represents a novel taxon with respect to the NCBI Genome database; highest taxonomic rank with p-value ≤ 0.01 are shown. Significance at p-value below: *0.5, ***0.05, ****0.01.*

$P < 0.001$; **Supplementary Figures 4, 5**). Results from the simulations were in line with the results of the regular model, and thus support that our results were not biased by the sampling design [PERMANOVA: Bray–Curtis, R^2 (min = 0.65, max = 0.98, mean = 0.78); P (min = 0.001, max = 0.019, $n < 0.05 = 10/10$); Jaccard, R^2 (min = 0.66, max = 1, mean = 0.86), P (min = 0.001, max = 0.106, $n < 0.05 = 5/7$)]. From all the additional factors examined, only host status (i.e., dead, alive) explained a significant amount of variance (**Table 2**). Including host status in PERMANOVA analyses did not alter the results on the major influence of host identity in explaining microbiome composition (PERMANOVA: Host identity, Bray–Curtis, $R^2 = 0.57$, $F = 4.58$, $P = 0.001$; Jaccard, $R^2 = 0.71$, $F = 5.09$, $P = 0.002$).

Similarly, in Kaiju matrices collapsed at the species level, NMDS ordinations showed a pattern of clustering by infrapopulation identity (**Figure 3B** and **Supplementary Figure 3B**). In the same vein, most (>80% in all cases) of the variance was also explained by infrapopulation identity (PERMANOVA: Bray–Curtis, $R^2 = 0.804$, $F = 4.346$, $P = 0.001$, **Figure 3B**; Jaccard, $R^2 = 0.803$, $F = 4.319$, $P = 0.001$; **Supplementary Figure 3B**). The analyses of differences in pairwise distances again supported the results: pairwise distances between samples from the same infrapopulations were lower than the median of among infrapopulation pairwise comparisons in 15 out of 17 infrapopulations (88%; Sign test: $P < 0.01$; **Supplementary Figures 5, 6**). Furthermore, results from simulations were similar [PERMANOVA: Bray–Curtis, R^2 (min = 0.62, max = 0.88, mean = 0.75); P (min = 0.003, max = 0.058, $n < 0.05 = 9/10$); Jaccard, R^2 (min = 0.63, max = 0.95, mean = 0.76), P (min = 0.002, max = 0.09, $n < 0.05 = 9/10$)]. Of all the other factors examined, only host status explained a significant amount of variance (**Table 2**). PERMANOVA analysis accounting for host status did not alter the relevance of host identity in explaining a significant amount of variance (PERMANOVA: Bray–Curtis, $R^2 = 0.52$, $F = 1.78$, $P = 0.007$; Jaccard, $R^2 = 0.59$, $F = 3.37$, $P = 0.001$).

Furthermore, results were consistent when using matrices collapsed at the genus level. Samples appeared clustered by infrapopulation identity in NMDS ordinations (**Supplementary Figures 7A,B**) and > 77% of variance was explained in all cases by this factor (PERMANOVA: Bray–Curtis, $R^2 = 0.865$, $F = 6.804$, $P = 0.001$, **Supplementary Figure 7A**; Jaccard, $R^2 = 0.774$, $F = 3.634$, $P = 0.001$; **Supplementary Figure 7B**). Once again, results from simulations were similar [PERMANOVA: Bray–Curtis, R^2 (min = 0.68, max = 0.96, mean = 0.8); P (min = 0.002, max = 0.073, $n < 0.05 = 9/10$); Jaccard, R^2 (min = 0.54, max = 0.86, mean = 0.73), P (min = 0.003, max = 0.061, $n < 0.05 = 9/10$)]. Additionally, of all the others factors examined, only host status explained a significant amount of variance [PERMANOVA: Bray–Curtis, Host status: $R^2 = 0.3$, $F = 14$, $P = 0.001$, Louse sex: $R^2 = 0.05$, $F = 0.51$, $P = 0.851$, Sequencing lane: $R^2 = 0.01$, $F = 0.39$, $P = 0.753$; Jaccard, Host-status: $R^2 = 0.18$, $F = 7.19$, $P = 0.002$, Louse sex: $R^2 = 0.07$, $F = 0.75$, $P = 0.53$, Sequencing lane: $R^2 = 0.01$, $F = 0.40$, $P = 0.75$; Mantel test, locality, Bray–Curtis: ρ (min = 0.09, max = 0.09, mean = 0.09), P (min = 0.720, max = 0.734, $n < 0.05 = 0/10$); Jaccard: ρ (min = 0.02, max = 0.02, mean = 0.02), P (min = 0.404, max = 0.425, $n < 0.05 = 0/10$)]. Likewise, PERMANOVA analysis accounting for host status did not alter the results on the relevance of host identity (PERMANOVA: Bray–Curtis, $R^2 = 0.56$, $F = 4.73$, $P = 0.001$; Jaccard, $R^2 = 0.59$, $F = 2.96$, $P = 0.001$).

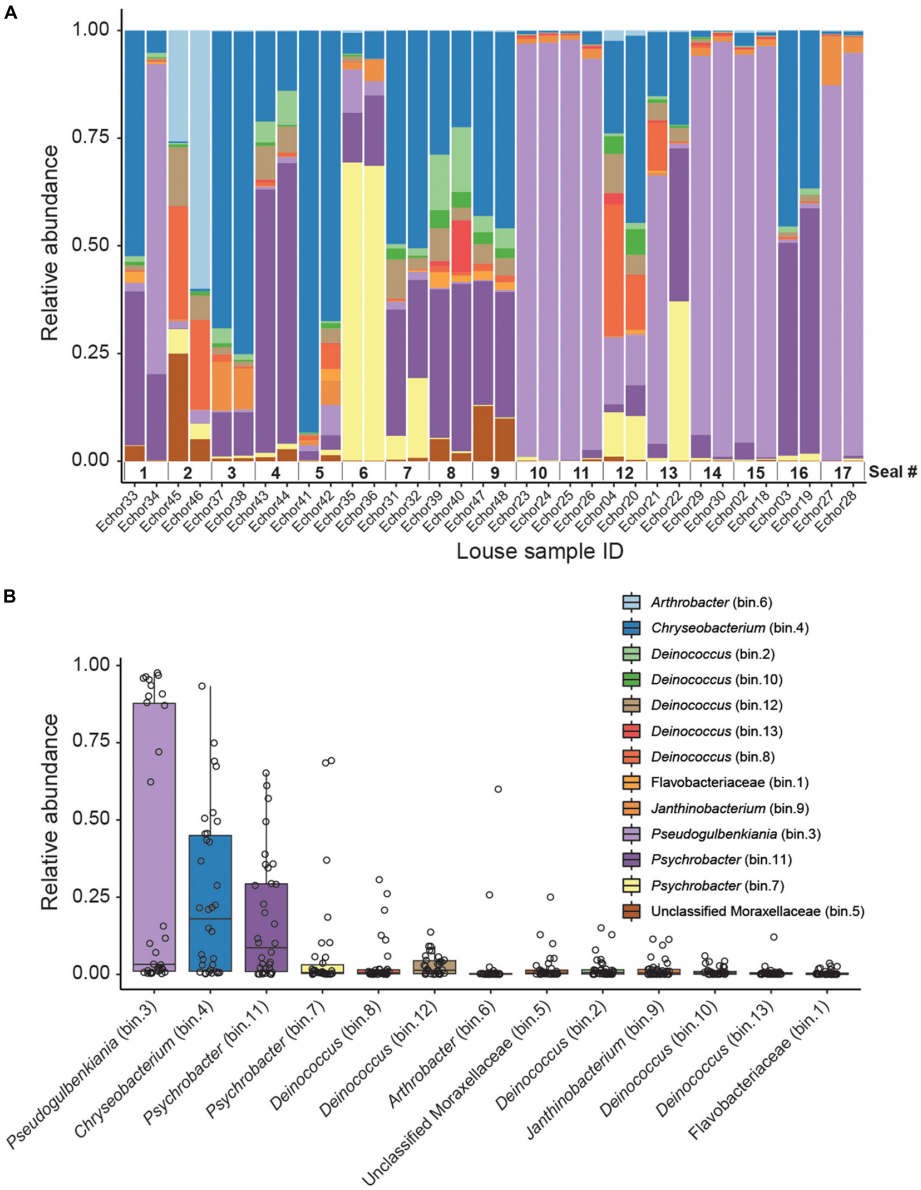

FIGURE 1 | Genome-resolved metagenomic data. **(A)** Stacked bar plot showing the relative abundances of MAGs in each louse sample. Note that samples are ordered according to host (i.e., samples from the same host are next to each other). **(B)** Boxplot summarizing the relative abundance of each MAG across the louse samples. Individual points (horizontally jittered) depict the relative abundance of each MAG in each sample.

DISCUSSION

Two different metagenomic approaches support a major role of infrapopulation identity (ringed seal host individual) in explaining microbiome variation among individuals of the seal louse. In addition, highly similar results were found for approaches using either presence–absence or quantitative matrices, suggesting that not only is bacterial composition, but also bacterial abundance explained by infrapopulation identity. Our analyses were done on whole louse individuals and, thus, we cannot confidently differentiate between bacterial taxa inhabiting the lice (e.g., *Wolbachia* or *Hodgkinia*) from transient taxa present in the host blood meal (e.g., *Chlamydia*). Nevertheless, in line with current evidence on the determinants of microbiome composition of bloodsucking parasites, the louse blood meal from individual seals is the most likely candidate in explaining the patterns of microbiome variation across the focal louse infrapopulations. Indeed, many of the taxa found in our analyses have already been found in other bloodsucking parasites, thus supporting the influence of blood in shaping the composition of parasite microbiomes studied here (Jiménez-Cortés et al., 2018).

However, other factors in addition to blood may have contributed to the similarity of microbiomes between individual lice from the same seal host individual. Some similarity may

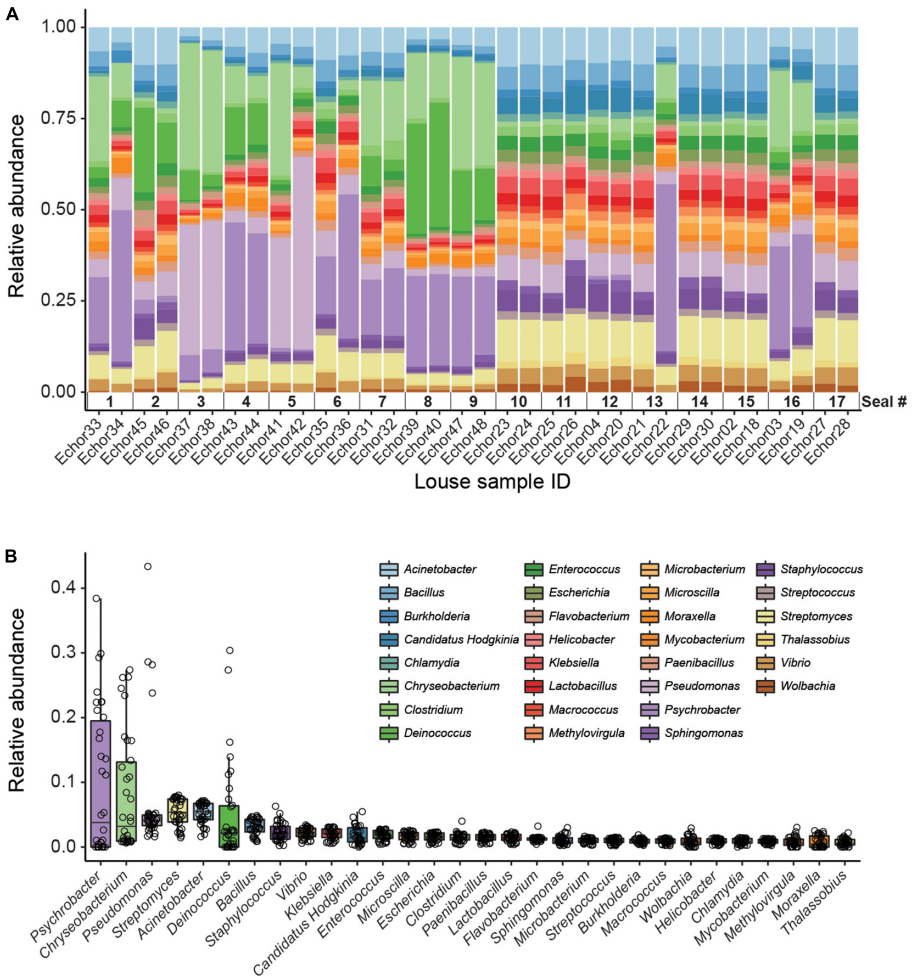

FIGURE 2 | Kaiju data (genus level). **(A)** Stacked bar plot showing bacterial relative abundances in each seal louse sample. Note that samples are sorted according to host individual (i.e., samples from the same host are next to each other). **(B)** Boxplot summarizing the relative abundance of each taxon across all louse samples. Individual points (horizontally jittered) depict the relative abundance of each taxon in each sample.

have arisen from shared environmental bacteria, i.e., those on the surface of the louse from a shared environment (skin and fur of the host), or contamination between louse individuals in screw-cap tubes, and not filtered by our decontamination procedures. While some potential contamination sources are nearly impossible to avoid, possible contamination between louse individuals in screw-cap tubes could have been avoided in this study should the louse from the same individuals have been placed in separate screw-cap tubes. We believe it seems unlikely, especially for some bacterial taxa (e.g., gut bacteria adhered to the gut epithelial cells; Narasimhan and Fikrig, 2015), that once in ethanol, these bacteria could have gone out of the louse individuals and reached the other louse interior. Nevertheless, our ethanol rinses, procedures to extract DNA (i.e., crashing whole louse individuals), and the bioinformatic decontamination filtering ensure this process does not mainly drive our results. There may also be insect-specific bacterial taxa, independent from the host blood, that are shared horizontally between individual lice from the same infrapopulation. Finally, louse infrapopulations are known to typically be highly inbred, with a high level of relatedness between individuals (Koop et al., 2014; DiBlasi et al., 2018; Virrueta Herrera et al., in prep.). It may be that genetic factors of the lice interact with the microbiome to produce a specific composition (Blekhman et al., 2015; Dobson et al., 2015; Suzuki et al., 2019).

Our results are congruent with previous findings on the influence of host blood on microbiomes of bloodsucking parasites. Specifically, several studies have found a major role of the specific host species from which a blood meal is taken in shaping microbiomes of other bloodsucking organisms, such as ticks (*Ixodes scapularis, Ixodes pacificus*) and mosquitoes (*Aedes aegypti*) (Swei and Kwan, 2017; Landesman et al., 2019; Muturi et al., 2019). Furthermore, Landesman et al. (2019) showed that microbiomes of deer tick (*Ixodes scapularis*) nymphs were largely explained by the individual hosts of the tick, a result similar to the one obtained here. Interestingly, in that study, the percentage of variation explained was considerably lower (45%) than that found here (>77%). It may be that differences in parasite ecology,

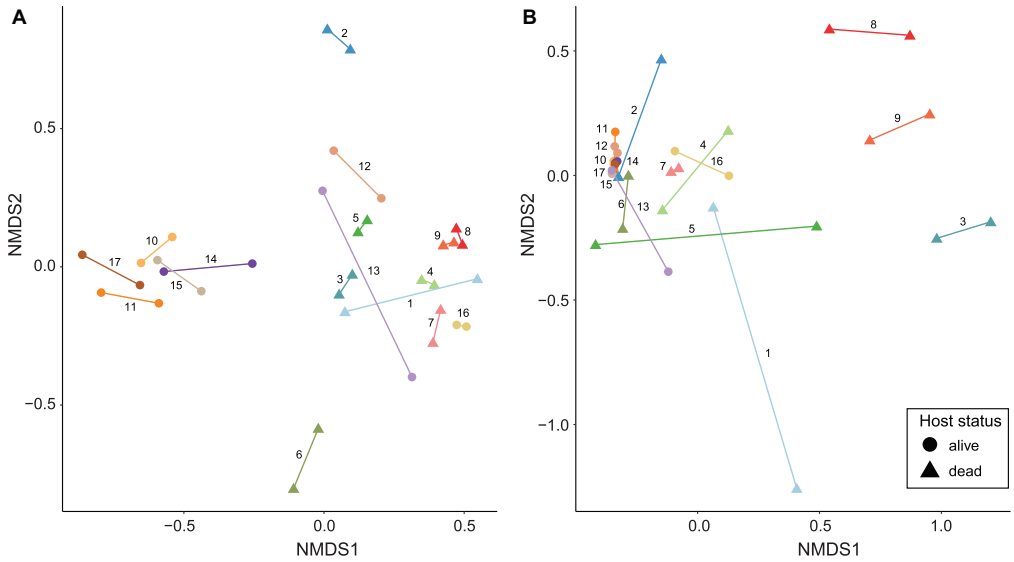

FIGURE 3 | NMDS ordinations of seal louse microbiomes based on Bray–Curtis dissimilarity matrices. **(A)** MAG matrix (stress = 0.132), and **(B)** Kaiju matrix (species level, stress = 0.081). Lice originating from the same seal individual are colored similarly and connected by a line, numbers next to lines refer to seal individuals in **Figures 1**, **2**.

TABLE 2 | PERMANOVA results from the factors in addition to infrapopulation that were evaluated to potentially influence microbiome variation among samples.

Data type	***MAGs***						***Kaiju***					
Dissimilarity distance	**Bray–Curtis**			**Jaccard**			**Bray–Curtis**			**Jaccard**		
Statistic Factor	**R^2/ ρ**	**F**	**P**	**R^2/ ρ**	**F**	**P**	**R^2/ ρ**	**F**	**P**	**R^2/ ρ**	**F**	**P**
Host status	0.28	12.72	0.001	0.13	4.93	0.002	0.22	9.03	0.001	0.21	8.73	0.001
Louse sex	0.08	0.9	0.554	0.03	0.28	0.867	0.08	0.81	0.564	0.08	0.88	0.497
Sequencing lane	0.01	0.38	0.878	0	0.01	1	0.01	0.35	0.859	0.01	0.4	0.825
Locality	−0.09; −0.09; −0.09	–	0.875; 0.887; 0	−0.29; −0.29; −0.29	–	0.97; 0.978; 0	0.04; 0.04; 0.04	–	0.564; 0.579; 0	−0.03; −0.03; −0.03	–	0.534; 0.549; 0

Note that the influence of locality was tested using multiple Mantel tests, and therefore ρ (min, max, and mean) and P (min, max, n < 0.05) simulation values are given (see section "Materials and Methods" for further details).

such as the whether the parasite is a permanent or a recurrent feeder (as are both the case in sucking lice) may modulate the extent to which host blood shapes parasite microbiomes. The differences in the proportion of variance explained by infrapopulation identity between the two studies could also be due to differences in experimental design, such as the number of sampled infrapopulations (3 in ticks, and 17 in the seal lice here) and whether the sample design is balanced (i.e., the same number of individual parasites sampled per infrapopulation).

The knowledge that intraspecific variation in the blood of seals may lead to similarity of the microbiomes of lice feeding on the same host individual can potentially provide new insights into the influence of host blood on parasites. At least two not necessarily mutually exclusive processes may explain the influence of a host individual's blood on louse microbiomes. First, the blood from a particular host individual may contain a specific composition of bacterial loads that enter the louse during feeding. Indeed, anopluran lice might have a high likelihood of being colonized by bacteria from host blood because they do not possess a peritrophic membrane, an extracellular layer in the midgut that is composed of chitin, proteoglycans, and proteins, which in most other insects surrounds the ingested food bolus and separates the gut content, including bacteria, from the epithelium (Terra, 2001; Waniek, 2009). Indeed, the idea that a lack of a peritrophic membrane facilitates colonization of blood-feeding parasites by bacteria present in the host blood has potentially also been supported by work on mouse fleas (*Rhadinopsylla dahurica*), which also lack this membrane (Li et al., 2018). In this case, there was evidence of homogenization (i.e., similar bacterial communities) between the host blood and the parasite (whole flea individuals). The lack of a peritrophic membrane is often associated with permanent parasites, such as blood-feeding lice, for which the continual availability of food means that there is less selection for efficient digestion. Therefore, the presence versus absence of a peritrophic membrane may explain the differences between lice and ticks (of which the latter possess a peritrophic membrane) with regards to the influence of host blood on the composition of the parasite microbiome.

A second possibility that could explain why host blood may influence louse microbiome composition is that the conditions

during blood digestion may alter bacterial taxa that were already present in the louse. The specifics of blood digestion may have an individual host-specific signature. Specifically, catabolism of blood meal leads to the generation of reactive oxygen species that are known to alter the midgut bacterial composition and diversity of bloodsucking parasites (Souza et al., 1997; Wang et al., 2011; Muturi et al., 2019). Also, the blood of different host species is known to differ in composition (e.g., total protein, hemoglobin, and hematocrit content), and these differences may lead to a differential proliferation of microbial taxa during digestion by parasites (Souza et al., 1997; Wang et al., 2011; Muturi et al., 2019). It may be the case that differences in blood composition among individuals even within the same host species may shape the bacterial composition of lice in a manner that is specific to host individuals.

Bloodsucking organisms, and anopluran lice in particular, are well known to rely on mutualistic endosymbionts to complement deficiencies in their diet (Perotti et al., 2008; Boyd and Reed, 2012; Boyd et al., 2017; Jiménez-Cortés et al., 2018). Notwithstanding that several of the bacterial taxa we found may not be stable inhabitants of lice, we found evidence for the presence of several louse-specific bacterial taxa (i.e., taxa that are highly unlikely to come from environmental contamination). These include the obligate intracellular arthropod bacteria *Wolbachia* (Werren, 1997) and *Hodgkinia* (for which only endosymbionts of cicadas are known; McCutcheon et al., 2009). Accordingly, we explored our MAGs for genome characteristics typical of endosymbionts. In particular, because endosymbiont genomes typically are small and have an AT bias, we explored the relative position of the observed MAGs in a "Genome size ∼ GC content" correlation plot (Wernegreen, 2015; **Figure 4**). Bin 1 appears to be the best candidate to be a mutualistic endosymbiont, according to its relative position in the correlation plot. This MAG was 100% complete (according to CheckM; Parks et al., 2015), detected in most samples (prevalence = 71%; similar endosymbiont prevalences have been found in Allen et al., 2016 and Říhová et al., 2019), and classified with confidence as Flavobacteriaceae. MiGA analyses suggest it may even belong to *Chryseobacterium* (*p*-value 0.585). Endosymbionts belonging to *Chryseobacterium* are known in other arthropods (e.g., termites, mosquitoes, cockroaches, and ticks; Eutick et al., 1978; Dugas et al., 2001; Campbell et al., 2004; Montasser, 2005; Burešová et al., 2006). Additionally, we conducted a preliminary investigation of the metabolic capabilities of this bacterium by investigating the completeness of metabolic pathways using GhostKOALA (Kanehisa et al., 2016) and KEGG-Decoder (Graham et al., 2018). This MAG has complete routes for synthesis of vitamin B (riboflavin), an essential amino acid (lysine), and several non-essential amino acids (e.g., serine; see **Supplementary Table 4**), as well as many fully or partially missing routes that may be redundant or potentially shared (or synthesized along) with the louse (**Supplementary Table 4**).

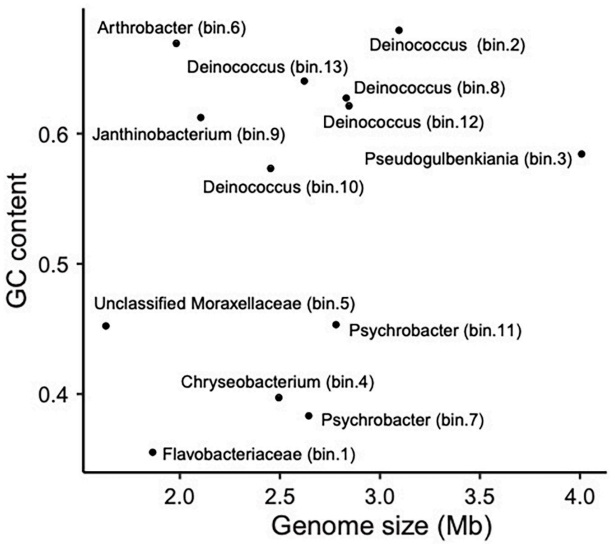

FIGURE 4 | Scatter plot showing the relationship between genome size (Mb) and GC content (i.e., proportion of G and C sites) for sequenced MAGs.

Overall, these results are congruent with what has been found for endosymbionts of bloodsucking parasites (Moriyama et al., 2015; Boyd et al., 2016; Santos-Garcia et al., 2017; Duron et al., 2018). Nevertheless, it is worth mentioning that the relative abundance of this putative endosymbiont MAG was the lowest among all the MAGs studied here. However, in contrast to what it is known for other bloodsucking parasites (e.g., ticks; Narasimhan and Fikrig, 2015), little is known in lice about the abundance and prevalence patterns of the microbiome of different organs and systems. In addition, apart from this study, whole-genome metagenomic data from different individuals are not available. Further research on the individual-level microbiomes of lice is needed to understand the relative abundances of bacteria in lice.

Another anopluran pinniped louse (*Proechinophthirus fluctus*) has been found to have a *Sodalis* endosymbiont (Boyd et al., 2016), but we found no evidence of *Sodalis* in *Echinophthirius horridus*. Other species of Anoplura have yet other endosymbionts (Boyd et al., 2017; Říhová et al., 2017, 2019), suggesting that endosymbiont replacement is an ongoing and relatively common process within the order. Population-scale studies are needed to better understand endosymbiont dynamics in populations of lice. Also, phylogenomic studies aiming at elucidating the phylogenetic placement of the potentially mutualistic *Chryseobacterium* found here and studies using fluorescence *in situ* hybridization (FISH) to ascertain its location in louse individuals are needed to get deeper insight into the interaction of this bacterium with *E. horridus*.

Lastly, the methodology used in this study (i.e., a dual metagenomic approach that combines genome-resolved metagenomics with metagenomic classification tools and state-of-the-art bioinformatic decontamination procedures) opens the door to further studies of the microbiomes of both parasites and free-living organisms for which WGS data are available. Here, this approach allowed us to characterize the variation of microbiomes among individuals of the same parasite species, and to identify factors underlying the observed variation. We were also able to identify potential endosymbionts and to

recover high-quality genomic data from them. Interestingly, in the current field of genomics, excessive accumulation of data and the resultant ever-increasing demand for data-storage capacity are worrying trends (Stephens et al., 2015). Thus, the possibility of using the same genome-level sequence datasets to address multiple different research questions (e.g., data generated for population-genomic analyses and cophylogenomics later leveraged to investigate introgression dynamics; Doña et al., 2020) and in different contexts (e.g., host-derived population-genomic data to infer bacterial composition; Hooper et al., 2019, this study) allows for more efficient use of existing genomic resources.

DATA AVAILABILITY STATEMENT

Raw sequence reads for all samples are available at SRA (see **Supplementary Table 1**). Metagenomic assemblies and FastQC reports (before and after preprocessing) are available at Figshare (doi: 10.6084/m9.figshare.12366575). Metagenomic assemblies are also available from NCBI Genome (SAMN18543074–SAMN18543086).

ETHICS STATEMENT

Telemetry studies have been approved by the local environmental authority Centre for Economic Development, Transport and the Environment (permit numbers: ESAELY/433/07.01/2012 and ESA-2008-L-519-254) and the Animal Experiment Board in Finland (permit numbers: ESAVI/8269/04.10.07/2013 and ESAVI-2010-08380/Ym-23).

AUTHOR CONTRIBUTIONS

JD, SV, and KJ conceived the study. TN and MK obtained samples. SV and KJ collected the data. JD analyzed the data and wrote the manuscript. TN, MK, and KJ obtained financial support for the project. All authors contributed to editing the manuscript.

FUNDING

This study was supported by the United States National Science Foundation (DEB-1239788, DEB-1342604, and DEB-1926919 to KJ). Sequencing costs were supported by grants from the Oskar Öflund Foundation, the Betty Väänänen Foundation, Societas Pro Fauna et Flora Fennica, and the Nestori Foundation.

ACKNOWLEDGMENTS

We thank all researchers and students who collected the lice analyzed in this study. Especially, we would like to thank researchers Miina Auttila, Vincent Biard, Meeri Koivuniemi, Lauri Liukkonen, Marja Niemi, Sari Oksanen, Mia Valtonen, and Eeva Ylinen for keeping an eye out for seal lice during their own studies on the Saimaa ringed seal.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.642543/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 03 September 2021
doi: 10.3389/fmicb.2021.695346

A *Rickettsiella* Endosymbiont Is a Potential Source of Essential B-Vitamins for the Poultry Red Mite, *Dermanyssus gallinae*

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *14 April 2021*
Accepted: *28 July 2021*
Published: *03 September 2021*

Citation:
Price DRG, Bartley K, Blake DP, Karp-Tatham E, Nunn F, Burgess STG and Nisbet AJ (2021) A Rickettsiella Endosymbiont Is a Potential Source of Essential B-Vitamins for the Poultry Red Mite, Dermanyssus gallinae. Front. Microbiol. 12:695346. doi: 10.3389/fmicb.2021.695346

Many obligate blood-sucking arthropods rely on symbiotic bacteria to provision essential B vitamins that are either missing or at sub-optimal levels in their nutritionally challenging blood diet. The poultry red mite *Dermanyssus gallinae*, an obligate blood-feeding ectoparasite, is a serious threat to the hen egg industry. Poultry red mite infestation has a major impact on hen health and welfare and causes a significant reduction in both egg quality and production. Thus far, the identity and biological role of nutrient provisioning bacterial mutualists from *D. gallinae* are little understood. Here, we demonstrate that an obligate intracellular bacterium of the *Rickettsiella* genus is detected in *D. gallinae* mites collected from 63 sites (from 15 countries) across Europe. In addition, we report the genome sequence of *Rickettsiella* from *D. gallinae* (*Rickettsiella* – *D. gallinae* endosymbiont; *Rickettsiella* DGE). *Rickettsiella* DGE has a circular 1.89 Mbp genome that encodes 1,973 proteins. Phylogenetic analysis confirms the placement of *Rickettsiella* DGE within the *Rickettsiella* genus, related to a facultative endosymbiont from the pea aphid and *Coxiella*-like endosymbionts (CLEs) from blood feeding ticks. Analysis of the *Rickettsiella* DGE genome reveals that many protein-coding sequences are either pseudogenized or lost, but *Rickettsiella* DGE has retained several B vitamin biosynthesis pathways, suggesting the importance of these pathways in evolution of a nutritional symbiosis with *D. gallinae*. *In silico* metabolic pathway reconstruction revealed that *Rickettsiella* DGE is unable to synthesize protein amino acids and, therefore, amino acids are potentially provisioned by the host. In contrast, *Rickettsiella* DGE retains biosynthetic pathways for B vitamins: thiamine (vitamin B1) *via* the salvage pathway; riboflavin (vitamin B2) and pyridoxine (vitamin B6) and the cofactors: flavin adenine dinucleotide (FAD) and coenzyme A (CoA) that likely provision these nutrients to the host.

Keywords: endosymbiont, mutualist, Gammaproteobacteria, vitamin biosynthesis, hematophagous, microbiome

INTRODUCTION

Animals live in a diverse bacterial world and mutualistic associations with bacteria can provide these animals with novel biochemical traits to exploit an otherwise inaccessible ecological niche (McFall-Ngai et al., 2013). For example, specialist phloem-feeding insects of the order Hemiptera depend on bacterial endosymbionts to synthesize and provide essential amino acids that are largely absent in their phloem sap diet (Moran, 2007). Similarly, obligate blood-feeding arthropods, including insects, ticks, and mites associate with nutritional mutualists that provide essential vitamins and cofactors that are in limited supply from their blood diet (recently reviewed in Husnik, 2018). Typically, as a result of relaxed selection, the genomes of nutritional mutualists are reduced relative to their free-living ancestors. Genes that are essential for symbiosis are retained, while non-essential genes are lost, resulting in small compact genomes (as reviewed by Toft and Andersson, 2010; McCutcheon and Moran, 2012). The microbiome of obligate blood-feeding invertebrates is often dominated by a single B vitamin provisioning symbiont. For example, the blood-feeding African soft tick (*Ornithodoros moubata*) is associated with a *Francisella* (strain F-Om) that provides the host with essential B vitamins to supplement its blood meal diet (Duron et al., 2018). The genome sequence of *Francisella* F-Om bears the hallmarks of a host-adapted bacterial endosymbiont, with dramatic genome reduction resulting from loss of redundant genes that are not required for a symbiotic function. Importantly, *Francisella* F-Om retains biosynthesis pathways for B vitamins biotin (B7), riboflavin (B2), folic acid (B9) and cofactors coenzyme A (CoA) and flavin adenine dinucleotide (FAD) to supplement deficiencies in the hosts diet (Duron et al., 2018). This pattern of genome reduction and retention of B vitamin biosynthesis pathways is also observed in *Coxiella*-like endosymbionts (CLEs) from obligate blood-feeding ticks. Recent genome sequence studies revealed that, in comparison to the pathogen *Coxiella burnetii* (genome size 2.03 Mbp), CLEs from ticks have reduced genomes, as small as 0.66 Mbp for CLE from the lone star tick (CLE of *Amblyomma americanum*). Yet, these nutritional mutualists retain pathways for B vitamin and cofactor biosynthesis to supplement the nutritional requirements of their blood-feeding host (Smith et al., 2015).

The poultry red mite (*Dermanyssus gallinae*) is an obligate blood-feeding ectoparasite that feeds on avian blood. This mite has a worldwide distribution and is endemic in many commercial poultry farms, with up to 83% of European egg-laying facilities infested by *D. gallinae* (George et al., 2015). Infestation of poultry houses has a serious impact on hen health and welfare and causes a significant reduction in both egg quality and production. Infestations can reach up to 500,000 mites per bird and cause welfare issues, including anemia, irritation, and even death of hens by exsanguination (Sigognault Flochlay et al., 2017). In the EU, *D. gallinae* infestation costs the poultry industry in excess of €231 million *per annum* due to production losses alone (Sigognault Flochlay et al., 2017). In addition, *D. gallinae* has been implicated in the transmission of avian viral and bacterial disease (Huong et al., 2014; Sigognault Flochlay et al., 2017).

To utilize blood as a food source, our current hypothesis is that *D. gallinae* associates with bacterial mutualists, which synthesize and supply essential B vitamins and cofactors that are absent in the blood diet of the mite. A previous microbiome analysis of *D. gallinae* demonstrates that adult female mites have a simple microbiome, with 10 operational taxonomic units (OTUs) accounting for between 90 and 99% of the observed microbial diversity (Hubert et al., 2017). Furthermore, only four bacterial taxa, including: *Bartonella*, *Cardinium*, *Wolbachia*, and *Rickettsiella*, were present across all *D. gallinae* life-stages (Hubert et al., 2017). Data presented here, based on bacterial 16S rRNA amplicon sequencing confirms the presence of *Rickettsiella* in *D. gallinae* eggs, in agreement with previous studies (De Luna et al., 2009; Hubert et al., 2017). Here, we investigate the distribution of the previously identified *Rickettsiella* – *D. gallinae* endosymbiont (*Rickettsiella* DGE; Hubert et al., 2017) in *D. gallinae* across Europe, determine the complete genome of *Rickettsiella* DGE and examine this genome for evidence of biosynthesis pathways, which would supplement the diet of its host, *D. gallinae*.

MATERIALS AND METHODS

Mite Collection and Endosymbiont-Enriched DNA Preparation

Dermanyssus gallinae were collected from a single commercial laying hen facility in the Scottish Borders, United Kingdom and maintained in 75 cm^2 canted tissue culture flasks (Corning Inc., Corning, NY, United States) at 4°C for up to 4 weeks after collection. For experiments requiring mite eggs, freshly collected mixed stage and gender mites were placed into vented 25 ml Sterilin universal tubes and maintained at 25°C, 75% relative humidity in a Sanyo MLR-350H incubator and eggs were collected the following day.

Since obligate bacterial endosymbionts are uncultivable outside the host, bacteria were derived from *D. gallinae* tissue lysates and host cells were removed from the extract using host depletion solution (Zymo Research, Irvine, CA, United States). Briefly, live mixed life-stage mites were surface sterilized with 70% (*v*/*v*) ethanol for 30 s at room temperature followed by three 1 min washes in nuclease-free water. Mites (approx. 25 mg) were then homogenized in 200 µl nuclease-free water using a tube pestle and host cells lysed by addition of 1 ml of host depletion solution (Zymo Research, Irvine, CA, United States) with a 15 min incubation at room temperature with end over end mixing. Intact bacterial cells were pelleted by centrifugation at 10,000 × *g* for 5 min at room temperature and DNA extracted from the pellet using a DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany). DNA concentration was assessed by the Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States) and 1% (*w*/*v*) agarose/TAE gel electrophoresis.

16S rRNA Amplicon Sequencing and Classification

Poultry red mite eggs were collected as described above using mites from the same commercial laying hen facility as described in section "Mite Collection and Endosymbiont-Enriched DNA

Preparation." Mite eggs were surface sterilized by two 5 min washes in 0.1% (*w*/*v*) benzalkonium chloride followed by two 5 min washes in 70% (*v*/*v*) ethanol. DNA was extracted from eggs using a DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany) with a lysozyme pre-treatment to lyse bacterial cells. DNA was quantified using a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and DNA molecular weight determined on a 1% (*w*/*v*) agarose/TAE gel. A reagent-only control DNA extraction was performed in parallel using the same DNA extraction kit.

The presence of bacterial DNA in mite eggs was verified by PCR using universal bacterial 16S rRNA gene primers 27F-short (5'-GAGTTTGATCCTGGCTCA-3') and 1507R (5'-TACCTTGTTACGACTTCACCCCAG-3'). Each 50 µl PCR reaction contained template DNA (100 ng), 1 U Platinum™ *Taq* DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, United States), 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and each primer at 0.2 µM. Cycling conditions were as follows: 94°C for 2 min; 30 cycles of 94°C 30 s, 58°C 30 s, 72°C 1 min 30 s, and a final hold of 72°C for 10 min. A control PCR reaction was performed using the same conditions with an equivalent volume of eluate from the reagent-only control extraction. PCR products were cloned into pJET1.2 using the CloneJet PCR cloning kit (Thermo Fisher Scientific, Waltham, MA, United States) and transformed into chemically competent JM109 *Escherichia coli* cells (Promega, Madison, WI, United States). Transformants were selected on Lysogeny broth (LB) agar plates containing 100 µg/ml ampicillin at 37°C. Colony PCR was performed on randomly selected individual colonies using pJET1.2-F (5'-CGACTCACTATAGGGAGAGCGGC-3') and pJET1.2-R (5'-AAGAACATCGATTTTCCATGGCAG-3') vector primers using the previously detailed cycling conditions, except the primer annealing temperature was reduced to 56°C. PCR products were analyzed on a 1% (*w*/*v*) agarose/TAE gel and colonies containing the expected size amplification product were grown overnight in 10 ml LB containing 100 µg/ml ampicillin at 37°C with shaking at 200 rpm. Plasmid DNA was isolated from each clone using Wizard® *Plus* SV Miniprep kit (Promega, Madison, WI, United States) and a total of 72 individual clones were sequenced with pJET1.2-F and pJET1.2-R primers at Eurofins Genomics Germany GmbH.

To assess the geographical association between *D. gallinae* and *Rickettsiella*, we used DNA from a previously published mite collection from 63 sites across Europe (Karp-Tatham et al., 2020). For each collection site, DNA extracted from a single mite was screened by PCR for the presence of *Rickettsiella*. Diagnostic *Rickettsiella* primers Rick-F (5'-GTCGAACGGC AGCACGGTAAAGACT-3') and Rick-R (5'-TCGGTTACCTT TCTTCCCCACCTAA-3') were designed based on *Rickettsiella* specific 16S rRNA regions using alignments in the PhylOPDb database (Jaziri et al., 2014). These primers were designed to amplify a 408 bp fragment of the *Rickettsiella* 16S rRNA gene. The diagnostic *Rickettsiella* 16S rRNA PCR primers were checked for specificity by running *in silico* searches against the current RDP 16S rRNA database (Wang et al., 2007). In addition, the specificity of the primers was validated by PCR on DNA isolated from adult female *D. gallinae* mites. Amplification products were analyzed by on a 1% (*w*/*v*) agarose/TAE gel to check for size and sequenced, confirming *Rickettsiella* specific amplification.

For screening European mite DNA, each 25 µl PCR reaction contained template DNA (5 ng), 0.5 U Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, United States), 1× PCR buffer, 0.2 mM of each dNTP and each primer at 0.5 µM. Cycling conditions were as follows: 98°C for 30 s; 30 cycles of 98°C 10 s, 68°C 30 s, 72°C 30 s, and a final hold of 72°C for 10 min. All PCR products were analyzed on a 1% (*w*/*v*) agarose/TAE gel and sequenced in both directions using Rick-seq-F (5'-AACGGCAGCACG GTAAAGAC-3') and Rick-seq-R (5'-AGTGCTTTACAACCC GAAGG-3') primers at Eurofins Genomics Germany GmbH.

16S rRNA sequences were classified with the RDP Classifier 2.13 (training set No. 18; Wang et al., 2007) and sequences with <80% bootstrap support as their genus assignment were removed from the dataset. All remaining sequences were used in blastn searches against the GenBank database to identify their top hit.

Genome Sequencing and Assembly

For the *Rickettsiella* genome assembly, we used PacBio reads from *D. gallinae* eggs that were generated in a previous study (Burgess et al., 2018). Sequence reads were derived from adult female mites collected at the same commercial laying hen facility as described in section "Mite Collection and Endosymbiont-Enriched DNA Preparation." The data set contained 7,318,092 reads for a total of 63,984,748,667 bases. Raw reads were mapped against the *D. gallinae* reference genome using Minimap2 v.2.17 (Li, 2018) and unmapped reads were extracted from the resulting BAM files using SAMtools v1.11 (Li et al., 2009). Unmapped reads (814,785 reads for a total of 1,274,422,647 bases) were assembled using the metaFlye assembler v.2.8.2 under default settings using the --pacbio-raw and --meta flags (Kolmogorov et al., 2020). The assembly containing 652 contigs was visualized with Bandage (Wick et al., 2015), which allowed identification of a circular 1.89 Mbp *Rickettsiella* genome with 12× coverage.

For massive parallel sequencing (MPS) host-depleted gDNA was extracted from mixed life-stage mites, *D. gallinae* mites collected from a commercial egg laying facility, as described in section "Mite Collection and Endosymbiont-Enriched DNA Preparation." DNA was fragmented using a Covaris system, size-selected for 200–400 bp fragments and used to construct a single strand DNA circle library. The library was amplified using phi29 DNA polymerase by rolling circle amplification to make DNA nanoballs (DNBs) and sequenced on a DNBSEQ-G50 platform as 150 bp paired end reads. Library construction and sequencing were performed by BGI Genomics (Shenzhen, China). This sequencing effort resulted in generation of 174,890,018 reads for a total of 26,233,502,700 bases. The reads were used to polish the *Rickettsiella* consensus sequence. Briefly, short-reads were mapped to the *Rickettsiella* genome using BWA-MEM aligner v0.7.17 (Li, 2013) and base calls were corrected using five iterative rounds of polishing with Pilon v1.23 (Walker et al., 2014).

The resultant assembly consisted of a single circular chromosome of 1,888,715 bp with 3,712× coverage.

Genome Annotation

The genome was annotated using Prokka v.1.14.6 (Seemann, 2014) and the automated pipeline included coding region prediction by Prodigal (Hyatt et al., 2010) and annotation of non-coding rRNAs using Barrnap and tRNAs using ARAGORN (Laslett and Canback, 2004). As part of the Prokka pipeline, insertion sequences (IS) were annotated using the ISfinder database (Siguier et al., 2006). Candidate pseudogenes were identified based on the length ratios of each predicted *Rickettsiella* DGE protein against their top blastp hit from searches against the NCBI nr protein database. *Rickettsiella* DGE proteins that deviated by +/− 25% compared to their top blastp hit were flagged as potential pseudogenes. Metabolic pathways for amino acids, B vitamins and cofactors were manually inspected using KEGG (Kanehisa and Goto, 2000) and MetaCyc (Caspi et al., 2006) reference pathways. The absence of genes in pathways was verified by tblastn searches against the *Rickettsiella* genome. The *Rickettsiella* DGE genome plot was generated using DNAplotter (Carver et al., 2009). Synteny analysis was performed between the *Rickettsiella* DGE and *Rickettsiella viridis* genome (accession number: AP018005) using MUMmer (Delcher et al., 2002; nucmer --maxgap=1,000 -mumreference -c 100).

Phylogenetic Analysis

A phylogenetic relationship of *Rickettsiella* isolates was reconstructed using 16S rRNA sequences obtained from *D. gallinae*. To reconstruct the *Rickettsiella* phylogeny, we retrieved additional 16S rRNA sequences from GenBank based on sequence similarity to the *Rickettsiella* DGE 16S sequence and more distantly related bacteria. This dataset included sequences from *Rickettsiella* found in various tick species, insect species, and other arthropods. Using this dataset 16S rRNA sequences were aligned using ClustalW (1,013 bp unambiguously aligned sites) and a maximum-likelihood (ML) phylogenetic tree constructed using the Kimura 2-parameter (K2) model with gamma distributed with invariant sites (G+I). The substitution model was selected based on BIC score (Bayesian Information Criterion) and reliability of the tree was tested using bootstrap analysis (1,000 replicates) with bootstrap values indicated on the tree. All phylogenetic analyzes were performed using MEGA version X (Kumar et al., 2018).

RESULTS AND DISCUSSION

Rickettsiella Is Present in *Dermanyssus gallinae* Eggs

16S rRNA amplicon sequencing of DNA isolated from a pool of surface-sterilized *D. gallinae* eggs reveals that *Rickettsiella* is detectable in mite eggs (**Figure 1**). From the 72 16S rRNA reads that were generated the majority of reads were from *Staphylococcus* sp. (56 reads, 78% of total reads), while the remainder were from *Rickettsiella* sp. (nine reads, 12.5% of total reads) and single reads (one read for each) to the following genera: *Blautia*; *Clostridium*; *Devosia*; *Paenalcaligenes*; *Salinicoccus*; *Streptococcus*; and *Tsukamurella* (**Figure 1**). Previous studies of the *D. gallinae* microbiome have identified *Rickettsiella* in all life-stages, including eggs, from mites collected from four geographically isolated commercial laying hen facilities in Czechia (Hubert et al., 2017). *Rickettsiella* is an obligate intracellular bacterium, therefore, it is not likely to be surface associated but found within cells of the mite egg, this raises the possibility that *Rickettsiella* is maternally inherited in *D. gallinae*.

Rickettsiella Infection Is Widespread in European Populations of *Dermanyssus gallinae*

We performed an extensive diagnostic PCR screen to test *D. gallinae* from collection sites throughout Europe for the presence of *Rickettsiella*. To do this, we used a previously produced *D. gallinae* DNA archive from mites sourced from commercial laying hen facilities from 63 sites across 15 European countries (Karp-Tatham et al., 2020). For each sample site, total *D. gallinae* DNA from a single adult mite was screened by diagnostic PCR using *Rickettsiella*-specific 16S rRNA primers. All *D. gallinae* DNA samples were *Rickettsiella* positive ($n = 63$), indicating that *Rickettsiella* infection is widespread in European *D. gallinae* populations (**Figure 2**). It is known that other animal- and plant-parasitic arthropods are associated with *Rickettsiella*. For example, non-pathogenic strains of *Rickettsiella* have been reported in the pea aphid *Acyrthosiphon pisum* (Tsuchida et al., 2010, 2014), leafhopper *Orosius albicinctus* (Iasur-Kruh et al., 2013), and ticks *Ixodes woodi* and *Ixodes uriae* (Kurtti et al., 2002; Duron et al., 2016). These strains of *Rickettsiella* are maternally inherited and can reach high frequencies in natural populations (Kurtti et al., 2002; Iasur-Kruh et al., 2013; Tsuchida et al., 2014; Duron et al., 2016).

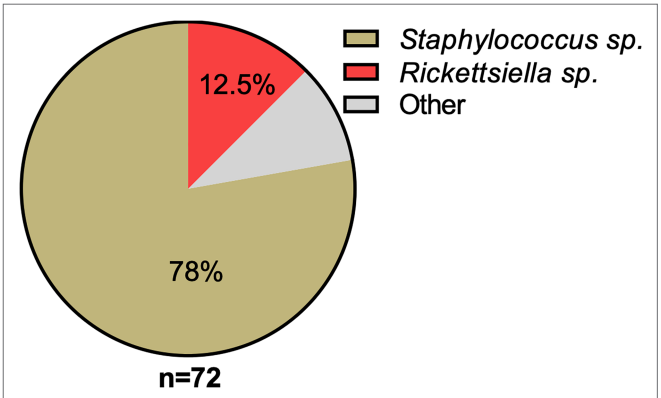

FIGURE 1 | Classification and relative abundance of bacteria associated with *Dermanyssus gallinae* eggs. The presence of bacterial DNA in mite eggs was verified by PCR using universal bacterial 16S rRNA gene primers. We generated a total of 72 16S rRNA reads from DNA isolated from surface-sterilized *D. gallinae* eggs. Amplicons were sequenced ($n = 72$) and classified with the RDP Classifier 2.13 (training set No. 18). Sequences with <80% bootstrap support as their genus assignment were removed from the dataset. Classifications are as indicated in the legend, other (gray) represents single hits ($n = 1$) to the following genera: *Blautia*, *Clostridium*, *Devosia*, *Paenalcaligenes*, *Salinicoccus*, *Streptococcus*, and *Tsukamurella*.

To date, most studies have focused on characterization of *Rickettsiella* population genetics and association with arthropods through sequence analysis of the16S rRNA gene. Therefore, to gain further insight into the biology of *Rickettsiella* from *D. gallinae*, we isolated DNA from mites and completed the *Rickettsiella* DGE genome sequence.

General Features of the *Rickettsiella* DGE Genome

We used previously-generated PacBio long-read sequence data from *D. gallinae* eggs (Burgess et al., 2018) to assemble the *Rickettsiella* DGE genome. From a total of 64.0 Gbp of sequence data, 1.3 Gbp of reads did not map to the *D. gallinae* draft genome and were used for metagenome assembly. The metagenome assembly contained 652 contigs and included a circular *Rickettsiella* DGE chromosome of 1.89 Mbp. To correct errors associated with long-read sequence data, the *Rickettsiella* DGE assembly was polished using five iterative rounds of Pilon with DNBSEQ™ short-read sequence data from symbiont enriched DNA. This yielded a circular chromosome of 1,888,715 bp with 3,712× coverage and a G+C content of 39.6% (**Figure 3**). Based on Prokka gene prediction and annotation, the *Rickettsiella* DGE genome has 1,973 protein coding open reading frames (ORFs) with an average size of 870 bp which covered 91% of the genome (**Table 1**; **Supplementary Table 1**). Of these ORFs, 970 were assigned a biological function by Prokka annotation, 585 were annotated by BLAST homology to characterized proteins, while 227 matched hypothetical proteins of unknown function and 191 were unique

FIGURE 2 | Map showing the distribution of *D. gallinae* populations analyzed in this study. All individual adult female *D. gallinae* mites from each sampling site (63 sites across Europe) were positive for *Rickettsiella* infection (red circle) suggesting that *Rickettsiella* infection may have reached fixation in European *D. gallinae* populations.

to *Rickettsiella* DGE. In seven cases, pairs of adjacent genes were annotated with identical names and clearly the ORF was interrupted by a stop codon splitting the gene into two or more parts (these genes are highlighted in **Supplementary Table 1**). It is likely that these fragmented genes are non-functional and in the early stages of pseudogenization. Other candidate pseudogenes were identified based on their length ratios of each predicted *Rickettsiella* DGE protein against their top blastp hit from searches against the NCBI nr protein database. In summary, out of a total of 1,973 *Rickettsiella* DGE protein coding ORFs searched, only 312 (15.8%) deviate by more than +/− 25% from their top hit and are candidate pseudogenes (**Supplementary Table 1**). However, it should be noted that the majority of these pseudogene candidates are "hypothetical proteins" of unknown function and, therefore, await experimental validation as genuine loss of function pseudogenes. We detected 41 tRNA genes (which can translate all 61 amino acid codons), six rRNA gene operons and 19 IS elements. *Rickettsiella* DGE contains 19 IS elements evenly distributed around the genome and there are eight copies of IS256 family transposase; four IS481; four ISNCY; and three IS5. Of these IS elements, two IS5 family elements have identical nucleotide sequences (OFBDPGAJ_01174 and OFBDPGAJ_01246) and seven IS256 family elements have identical nucleotide sequences (OFBDPGAJ_00304; OFBDPGAJ_00358; OFBDPGAJ_00392; OFBDPGAJ_00512; OFBDPGAJ_01167; OFBDPGAJ_01364; and OFBDPGAJ_01423). Due to high sequence similarity, it is likely that these IS elements are recently active in the *Rickettsiella* DGE genome.

Rickettsiella DGE Is Related to Endosymbionts and Endoparasites From the Order *Legionellales*

Our phylogenetic analysis, using 16S rRNA gene sequences from representative Gammaproteobacteria, supports the placement *Rickettsiella* DGE within the *Rickettsiella* genus (**Figure 4**).

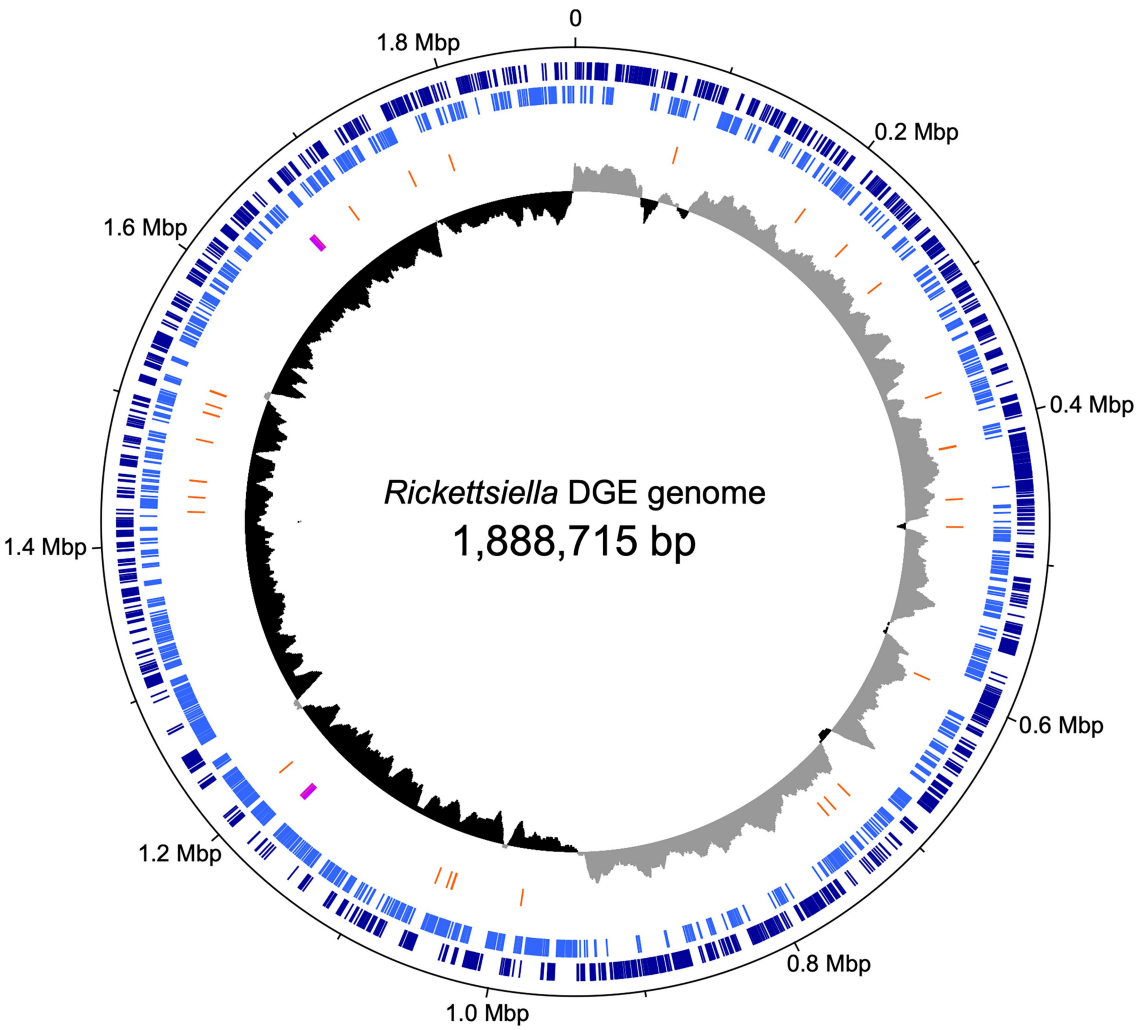

FIGURE 3 | Map of the circular chromosome of *Rickettsiella* DGE. The innermost circle shows GC skew (window size: 10,000 bp) with gray and black indicating high (>0) and low (<0) (G−C)/(G+C) values. The second circle shows the positions of tRNA genes (orange) and rRNA genes (purple). The outer circles indicate the positions of protein coding genes on the plus strand (dark blue) and minus strand (light blue).

TABLE 1 | General genomic features of *Rickettsiella* DGE and allied Gammaproteobacteria.

	Rickettsiella DGE	*R. viridis*	*Coxiella burnetii* RSA 493	*Escherichia coli* K-12
Genome size, Mbp	1.89	1.58	2.00	4.64
G+C (%)	39.6	39.3	42.7	50.8
Protein-coding genes	1,973	1,362	1,798	4,242
Number of COGs[#]	1,322	1,033	1,293	3,812
Coding density (%)	91.0	87.1	77.7	85.8
Average gene size	870	1,010	862	939

[#]*COG, cluster of orthologous groups.*

Members of the *Rickettsiella* genus form a monophyletic group that diverged from *C. burnetii*, the etiologic agent of Q fever, approximately 350 million years ago (Cordaux et al., 2007). *Rickettsiella* sp. are found in a wide range of arthropod hosts and are best known as obligate intracellular pathogens (Cordaux et al., 2007; Leclerque and Kleespies, 2008), though, recently, some have been characterized as mutualistic endosymbionts (Tsuchida et al., 2010; Duron et al., 2015). Based on phylogenetic analysis using 16S rRNA sequences, *Rickettsiella* DGE is closely related to *Rickettsiella* that was isolated from *D. gallinae* from commercial egg laying facilities in Czechia (Hubert et al., 2017). Furthermore, all *Rickettsiella* strains from *D. gallinae* are closely related to *Rickettsiella* of the tick *I. uriae* (Duron et al., 2016) and *R. viridis* of the pea aphid, *A. pisum* (Nikoh et al., 2018; **Figure 4**). In aphids, *R. viridis* infection is associated with production of blue-green pigment molecules that accumulate in the host (Tsuchida et al., 2010) and not associated with negative impacts on host fitness (Tsuchida et al., 2010). As *Rickettsiella* present in *D. gallinae* is closely related to strains found in other species it may indicate horizontal transfer of *Rickettsiella* across arthropod species (**Figure 4**). Whole genome alignments reveal shared synteny between *Rickettsiella* DGE and *R. viridis*, with evidence of genomic rearrangements including inversions, translocations, and insertions (**Figure 5**).

Rickettsiella DGE is related to other nutritional endosymbionts of blood-feeding arthropods in the order Legionellales. Within the order Legionellales, the CLEs of ticks form a monophyletic group most closely related to the human pathogen *C. burnetii* (**Figure 4**). In blood-feeding ticks, CLEs are required for the synthesis and supplementation of B vitamins that are lacking in the host's blood meal and are essential for tick survival (Guizzo et al., 2017). In addition, and again within the order Legionellales, the blood-feeding louse *Polyplax serrata* is associated with a vertically transmitted, host restricted, nutritional endosymbiont from the genus *Legionella* (Říhová et al., 2017; **Figure 4**). In *P. serrata*, these endosymbionts synthesize and provision B vitamins to their obligate blood-feeding host (Říhová et al., 2017). In summary, endosymbiotic bacteria from the order Legionellales are widely associated with blood feeding arthropods.

Genomic Reduction in *Rickettsiella* DGE: An Ongoing Process?

Genome reduction is widespread in maternally-inherited bacterial endosymbionts and is associated with loss of genes that are functionally redundant with the host, resulting in compact endosymbiont genomes containing a subset of genes relative to their free-living ancestor (McCutcheon and Moran, 2012). The genome of *Rickettsiella* DGE (1.89 Mbp) and *R. viridis* (1.6 Mbp) are both moderately reduced in comparison to *C. burnetii* (2.03 Mbp; **Table 1**). Although, it should be noted that *C. burnetii* is already host-adapted as an obligate intracellular parasite and, as such, compared to free-living bacteria it has a degenerate genome (Seshadri et al., 2003). Again, relative to *C. burnetii*, CLEs of blood-feeding ticks have reduced genomes, retaining functionally non-redundant genes that are essential for the symbiosis. Recent genome sequencing studies unveiled that, in comparison to *C. burnetii* (genome size 2.03 Mbp), CLEs from ticks exhibit genome reduction, with genomes ranging from 0.66 Mbp for *Coxiella* sp. strain CLEAA (CLE of *A. americanum*; Smith et al., 2015) to 1.73 Mbp for *Coxiella* sp. strain CRt (CLE of *Rhipicephalus turanicus*; Gottlieb et al., 2015). Presumably the range of genome size among CLEs of blood-feeding ticks reflects an ongoing dynamic process of reductive genome evolution. Metabolic reconstruction of these reduced genomes reveals intact B vitamin biosynthesis pathways, required for biosynthesis and provision of these essential nutrients to the host tick (Gottlieb et al., 2015; Smith et al., 2015).

Perhaps the most striking example of genome reduction, in the transition from a pathogen to a nutritional mutualist, is the loss of virulence associated secretion systems. In the pathogens *C. burnetii* and *Legionella pneumophila* the type IV Dot/Icm secretion system (T4SS) functions to export a suite of virulence factors that modulate host physiology and are essential for establishment and maintenance of infection (Seshadri et al., 2003; Chien et al., 2004; Gomez-Valero et al., 2019). Intriguingly, the massively reduced genomes of *Coxiella* from the lone star tick *A. americanum* (CLEAA) and *Legionella polyplacis* from the blood-feeding louse *P. serrata* do not encode a Dot/Icm type IVB secretion system and presumably this secretion apparatus is not required in these nutritional mutualists (Smith et al., 2015; Říhová et al., 2017). In contrast, components of the Dot/Icm type IVB secretion system are retained in *Rickettsiella* DGE and are also present in the closely related genome of *R. viridis*, although, the sequences of core components are highly divergent when compared with *L. pneumophila* orthologs (**Supplementary Table 2**). It therefore remains to be determined if the Dot/Icm type IVB secretion system is functional in *Rickettsiella* DGE and the role it plays in cellular interactions with the host.

Metabolic Capacity of *Rickettsiella* DGE: A Putative Nutritional Mutualist

The *Rickettsiella* DGE genome, as with the related intracellular facultative symbiont *R. viridis*, retains genes for basic cellular processes including translation, replication, cell wall biosynthesis, and energy production (**Figure 6**). In **Supplementary Table 3**, we provide a more detailed comparative gene content analysis

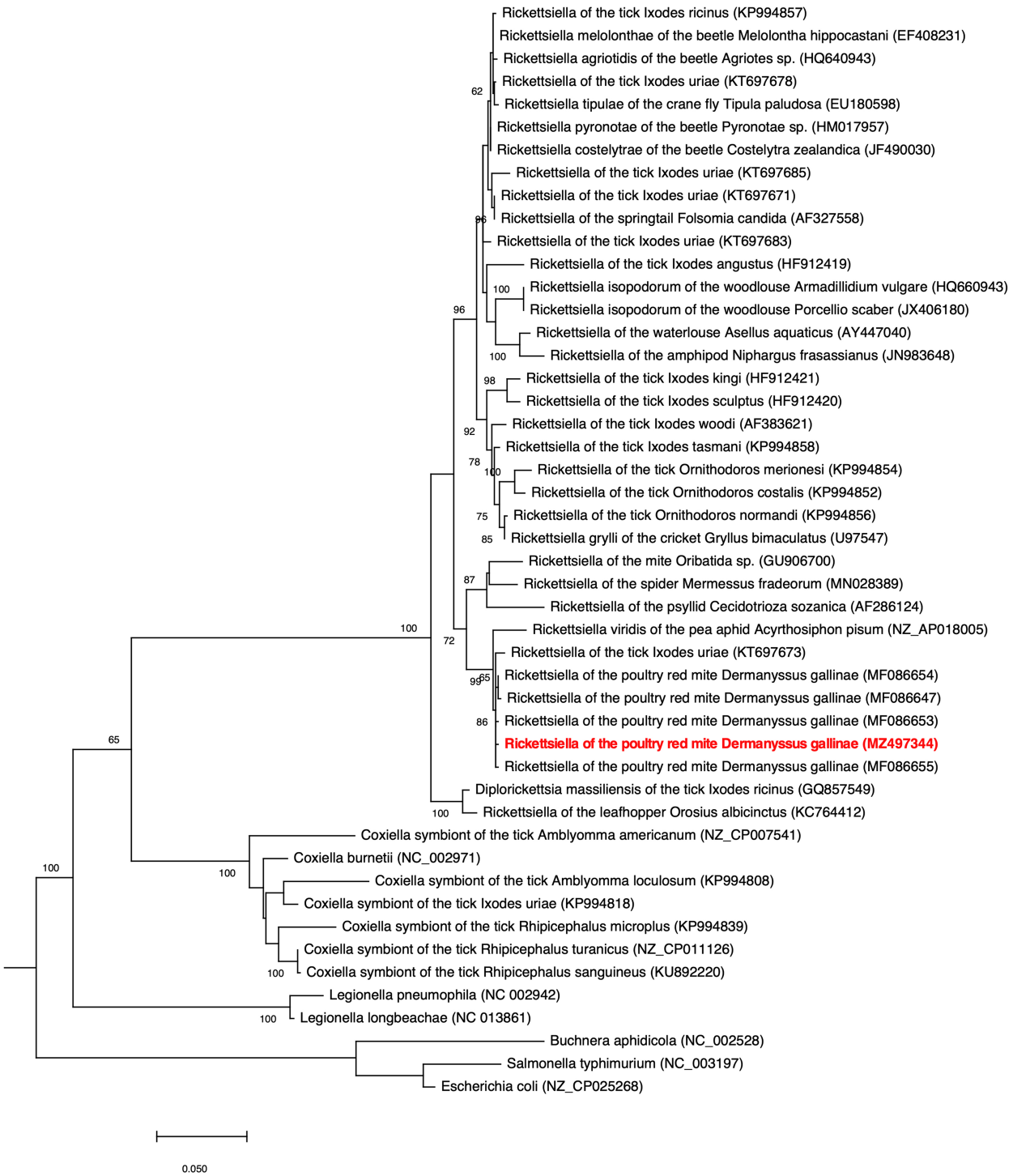

FIGURE 4 | Phylogenetic placement of *Rickettsiella* DGE in the Gammaproteobacteria. The maximum likelihood phylogeny is inferred from 16S rDNA sequences (1,013 unambiguously aligned nucleotide sites). Statistical support is shown at each node from 1,000 bootstrap replicates (bootstrap values > 60% are shown). The *Rickettsiella* DGE sequence highlighted in red (MZ497344) was generated in the current study. Accession numbers are indicated in brackets. Scale bar represents 0.02 substitutions per site.

between *Rickettsiella* DGE and genomes of *R. viridis* and *C. burnetii* using the pathway/gene list published by Moran et al. (2008) and Bennett and Moran (2013).

Metabolic reconstruction of amino acid biosynthesis pathways revealed that *Rickettsiella* DGE is unable to synthesize protein amino acids and, therefore, amino acids are likely provisioned

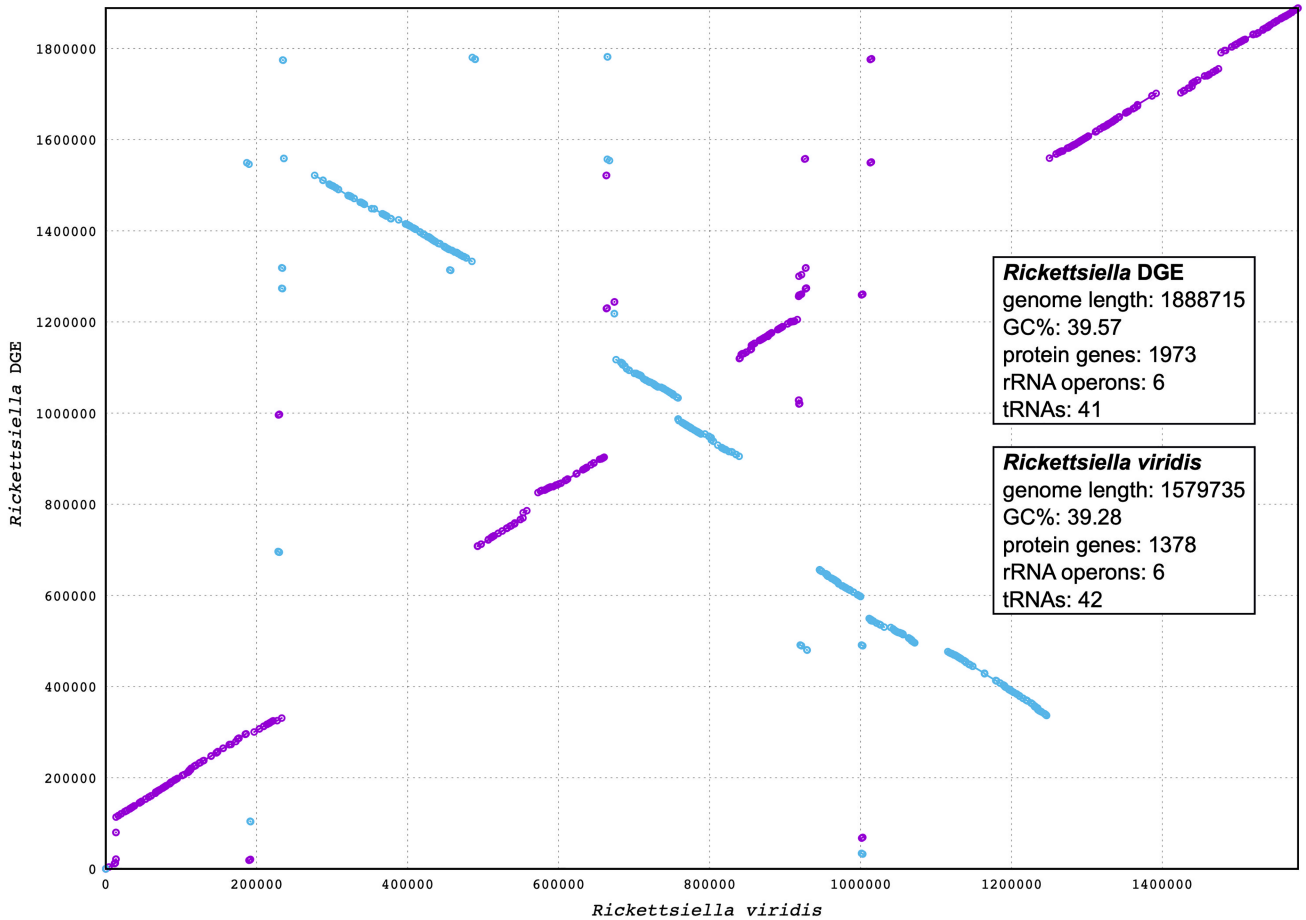

FIGURE 5 | Synteny analysis between *Rickettsiella* DGE and *Rickettsiella viridis* genomes. The *Rickettsiella* DGE genome is represented on the *y* axis and the *R. viridis* genome is represented on the *x* axis. Blue and purple lines represent synteny between the two genomes, with blue lines being inverted in *Rickettsiella* DGE relative to *R. viridis*.

by the host (**Figure 7**). The biosynthesis pathway for the essential amino acid lysine is mostly complete (8/9 required genes present), although, precursor aspartic acid in not synthesized by *Rickettsiella* DGE and the bifunctional aspartokinase/homoserine dehydrogenase 1 (encoded by *thrA*) is missing, again suggesting this pathway is non-functional. Given that *D. gallinae* feeds on blood and is able to digest hemoglobin and other blood proteins to release free amino acids (Price et al., 2019), it likely has an excess of essential and non-essential amino acids that meet its own nitrogen requirements and those of *Rickettsiella* DGE. Indeed, in other nutritional endosymbionts of obligate blood feeding arthropods, amino acid biosynthesis pathways are absent and it is likely the host supplies amino acids to the endosymbiont (Chien et al., 2004; Smith et al., 2015; Duron et al., 2018).

Obligate blood feeding arthropods such as the human body louse (*Pediculus humanus*; Kirkness et al., 2010), African soft tick (*O. moubata*; Duron et al., 2018) and the Lone star tick (*A. americanum*; Smith et al., 2015) depend on nutritional endosymbionts to synthesize and provide B vitamins that are available in trace amounts in mammalian blood (reviewed in Husnik, 2018). Thus, to determine whether *Rickettsiella* DGE is able to synthesize B vitamins, we surveyed its genome for B vitamin biosynthesis genes. The *Rickettsiella* DGE genome has conserved genes involved in the biosynthesis of seven B vitamins, including complete biosynthetic pathways for thiamine (vitamin B1) *via* the salvage pathway, riboflavin (vitamin B2), pyridoxine (vitamin B6) and the cofactors FAD, and CoA (**Figure 7**). The biosynthesis pathway for biotin (vitamin B7) is largely complete (9/10 genes present), although, it is missing *bioH*, which is required for pimeloyl-CoA synthesis. The annotated biotin biosynthesis pathway is based on that of the model organism *E. coli*, where *bioC* and *bioH* are required for synthesis of the intermediate pimeloyl-CoA. However, unlike the representative "*bioC*/*bioH*" pathway of *E. coli* many *bioC*-containing microorganisms lack *bioH* homologs, raising the possibility of non-homologous gene replacement in some bacteria (Shapiro et al., 2012). To date, there are five documented cases of *bioH* gene replacement, which includes *bioK* of *Synechococcus* (Shapiro et al., 2012), *bioG* of *Haemophilus influenzae* (Shapiro et al., 2012), *bioJ* of *Francisella* sp. (Feng et al., 2014), *bioV* of *Helicobacter* sp. (Bi et al., 2016), and *bioZ* of *Agrobacterium*

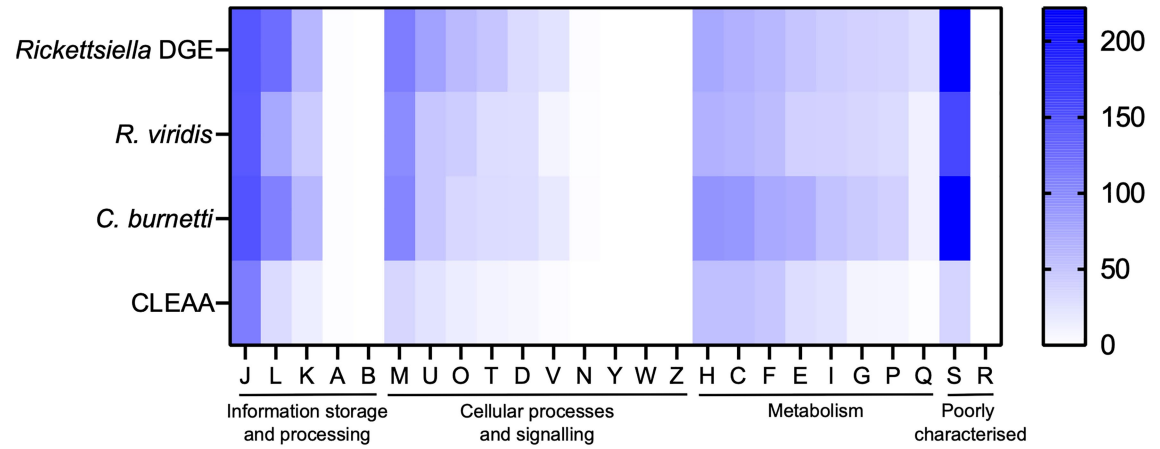

FIGURE 6 | Heatmap comparison of cluster of orthologous groups (COG) frequency in *Rickettsiella* DGE and related bacteria. Abbreviations for functional categories are as follows: J, translation, ribosomal structure, and biogenesis; L, replication, recombination, and repair; K, transcription; A, RNA processing and modification; B, chromatin structure and dynamics; M, cell wall/membrane/envelope biogenesis; U, intracellular trafficking, secretion, and vesicular transport; T, signal transduction mechanisms; O, posttranslational modification, protein turnover, chaperones; D, cell cycle control, cell division, chromosome partitioning; V, defense mechanisms; N, cell motility; Y, nuclear structure; W, extracellular structures; Z, cytoskeleton; H, coenzyme transport and metabolism; C, energy production and conversion; F, nucleotide transport and metabolism; E, amino acid transport and metabolism; I, lipid transport and metabolism; G, carbohydrate transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport, and catabolism; S, function unknown; and R, general function prediction only. Scale bar (0, white; 200, blue) indicates number of COGs in each category.

tumefaciens (Hu and Cronan, 2020). Further tblastn searches against the *Rickettsiella* DGE genome using *bioH* and the non-homologous gene replacements *bioK*, *bioG*, *bioJ*, and *bioV* did not identify gene products that can fill the *bioH* gap. However, a gene encoding ketoacyl-ACP synthase (KAS) III from *Rickettsiella* DGE (gene locus OFBDPGAJ_01014) has similarity to *bioZ* of *A. tumefaciens* (53.8% amino acid similarity) and is therefore a candidate to replace *bioH*. Alignments between *A. tumefaciens* KAS III (*bioZ*) and orthologs from *Rickettsiella* DGE as well as other *Rickettsiella* sp. are shown in **Supplementary Figure 1**. Given the retention of a long biotin biosynthesis pathway in *Rickettsiella* DGE (9/10 genes present) and the propensity for the missing *bioH* gene to be replaced in other bacteria, we predict that the biotin biosynthesis pathway is functional in *Rickettsiella* DGE. In contrast, the other B vitamin biosynthesis pathways for nicotinic acid (vitamin B3), pantothenic acid (vitamin B5), and folic acid (vitamin B9) are more fragmented and it is not clear if these pathways are functional.

In other nutritional host/endosymbiont interactions it has been shown that some fragmented metabolic pathways of nutritional endosymbionts are functional with gene products supplemented from multiple species including the host and/or symbiont partners. This complex arrangement results in metabolic mosaics for the synthesis of essential nutrients (McCutcheon et al., 2009; Husnik et al., 2013). By utilizing the *D. gallinae* genome (Burgess et al., 2018), we investigated whether host gene products are capable of filling missing steps in *Rickettsiella* DGE B vitamin biosynthesis pathways. In general, animals cannot synthesize B vitamins *de novo*, therefore, we explored the possibility that *D. gallinae* has acquired genes through horizontal gene transfer (HGT) that allows these fragmented pathways to function. To screen for potential HGT events, we used *E. coli* proteins from each of the missing steps in *Rickettsiella* DGE B vitamin biosynthesis as "query" proteins in blastp searches against predicted proteins from the *D. gallinae* genome (Burgess et al., 2018). These searches did not identify candidate genes from *D. gallinae* and it is therefore unlikely that *D. gallinae* contributes to B vitamin biosynthesis by completing these missing steps. Another possibility is that fragmented B vitamin pathways in *Rickettsiella* DGE are completed by gene products from other endosymbionts of the mite. A previous microbiome analysis of *D. gallinae* identified several additional endosymbionts (including *Bartonella*, *Cardinium*, and *Wolbachia*) that are prevalent in mite populations (Hubert et al., 2017). However, the biosynthetic capability of these *D. gallinae* endosymbionts is currently unknown (Hubert et al., 2017). Thus, future work will analyze B vitamin biosynthesis in the context of the *D. gallinae* metagenome.

In addition to blood feeding ticks and mites, many insects are specialist blood feeders (reviewed in Husnik, 2018). To utilize their blood diet, obligate blood feeding insects also associate with mutualistic endosymbionts that are important for provision of B vitamins to the host (Akman et al., 2002; Kirkness et al., 2010; Nikoh et al., 2014). While endosymbiotic partners differ, there are many commonalities between endosymbiotic partners from blood-feeding ticks, mites, and insects (Husnik, 2018). For example, the tsetse fly (*Glossina morsitans*) is critically reliant on its obligate endosymbiont *Wigglesworthia glossinidia*. Elimination of the symbiont using antibiotic treatment results in reproductive failure of the tsetse host (Rio et al., 2016). Critically, it has been shown that reproduction can by partially restored in these flies by dietary supplementation with B vitamins, suggesting that the

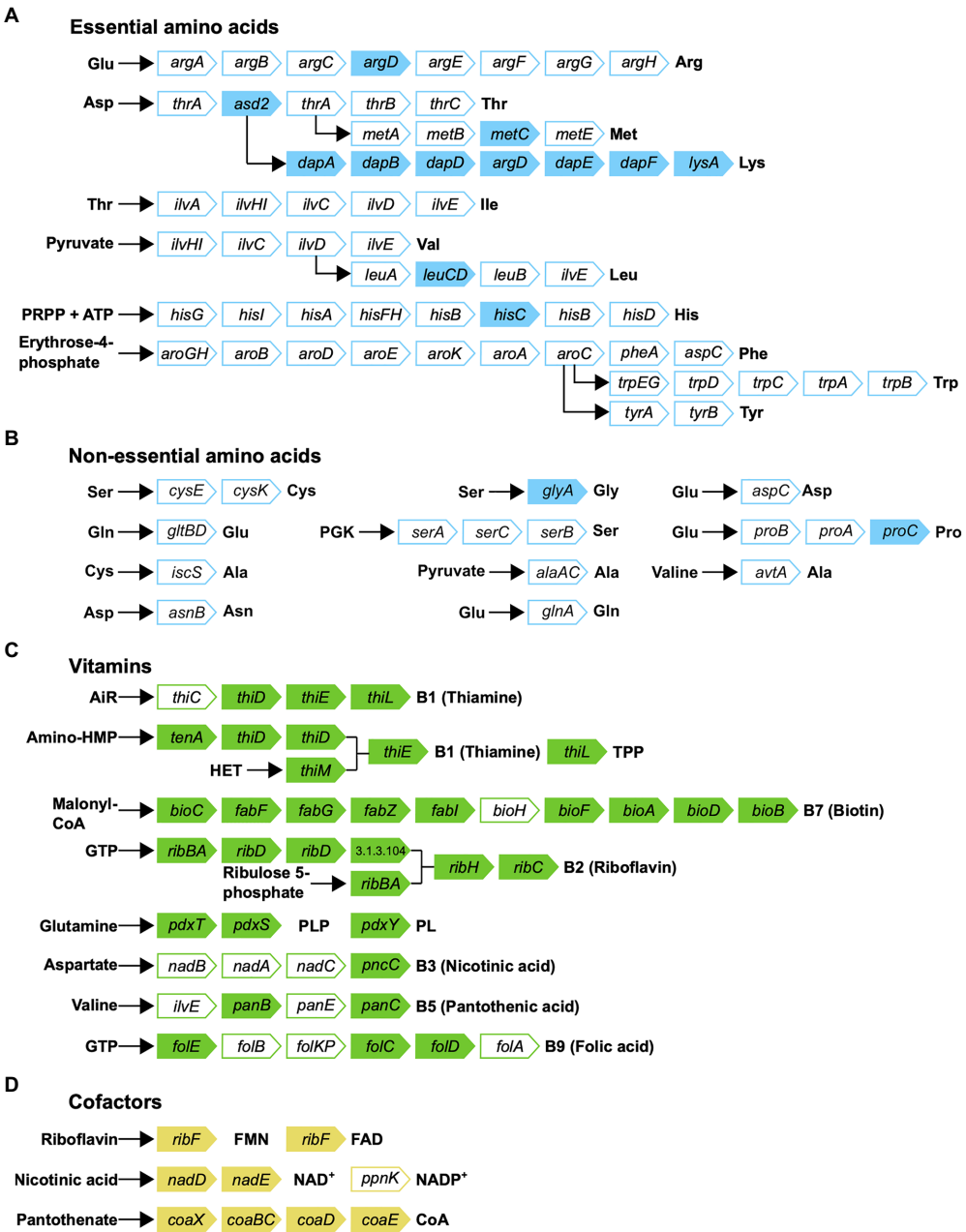

FIGURE 7 | Biosynthetic pathways for synthesis of **(A)** essential amino acids; **(B)** non-essential amino acids; **(C)** vitamins; and **(D)** cofactors in *Rickettsiella* DGE. Gene names are indicated in arrowed rectangles; colored arrows show genes present in *Rickettsiella* DGE; missing genes are shown in white arrows.

endosymbiont may provision these nutrients (Rio et al., 2016). Furthermore, in support of its role as a nutritional mutualist, the genome of *Wigglesworthia* reveals that the small 700 Kbp endosymbiont genome retains the capability to synthesise B vitamins (Akman et al., 2002; Rio et al., 2012). In comparison to *Rickettsiella* DGE, where we observe several incomplete B vitamin biosynthesis pathways (**Figure 7**), *Wigglesworthia* has complete pathways for the synthesis of biotin (vitamin B7), thiamine (vitamin B1), riboflavin (vitamin B2), pantothenic acid (vitamin B5), and pyridoxine (vitamin B6; Akman et al., 2002; Rio et al., 2012). Although speculative, this may reflect differing requirements of the host for B vitamin supplementation across these host/endosymbiont systems. Indeed, genome analysis of endosymbionts from other blood feeding arthropods reveals differing levels of completeness of retained B vitamin biosynthesis pathways (Kirkness et al., 2010; Nikoh et al., 2014; Smith et al., 2015; Duron et al., 2018). A recent analysis of endosymbiont genomes from obligate blood feeding arthropods reveals that all genomes analyzed retain "core" biosynthesis pathways for biotin (vitamin B7) and to a lesser degree folic acid (vitamin B9)

and riboflavin (vitamin B2; Duron and Gottlieb, 2020). In the analysis by Duron and Gottlieb (2020), other B vitamin pathways were more fragmented and pathway functionality may reflect the lifestyle of the host and its nutritional requirement for B vitamin supplementation. In both *Rickettsiella* DGE and other endosymbionts from blood feeding arthropods further investigation is needed to understand if and how these fragmented B vitamin biosynthetic pathways are functional as well as the exact B vitamin requirements of each host.

Currently, in the *D. gallinae* – *Rickettsiella* DGE endosymbiotic system the tissue location of *Rickettsiella* DGE is unknown, as is the identity of host genes required for the maintenance of the association. Again, this is something that has been extensively investigated in the tsetse/*Wigglesworthia* interaction (Bing et al., 2017). In teste flies, *Wigglesworthia* is located in host bacteriocyte cells that collectively form the bacteriome organ in the anterior midgut (Rio et al., 2012). Dual analysis of the host/endosymbiont transcriptome identified host factors that contribute to the maintenance of the symbiosis and a multivitamin transporter potentially involved in nutrient provision to the host (Bing et al., 2017). In support of its role as a nutritional mutualist, genes involved in biosynthesis of B vitamins and co-factors were highly expressed by the endosymbiont (Bing et al., 2017). Thus, the key priorities for future research are to determine the molecular processes underpinning maintenance of *Rickettsiella* DGE in host cells and the genetic and metabolic mechanisms by which nutrient flux between host and endosymbiont is regulated.

DATA AVAILABILITY STATEMENT

DNBseq reads were deposited to the Sequence Read Archive (SRA), under NCBI BioProject PRJNA743410. The genome sequence of *Rickettsiella* DGE has been deposited at GenBank with the accession number CP079094. *Rickettsiella* DGE 16S rRNA generated in this study is available from GenBank under the following accession numbers: MZ497336–MZ497344.

AUTHOR CONTRIBUTIONS

DP, AN, and SB conceived the study and analyzed the data. DP, KB, DB, EK-T, FN, SB, and AN designed the research. DP and EK-T performed the research. DP wrote the paper with contributions from all authors. All authors contributed to the article and approved the submitted version.

FUNDING

The work was supported by a Moredun Foundation Research fellowship awarded to DP and a British Egg Marketing Board (BEMB) Trust PhD scholarship awarded to EK-T.

ACKNOWLEDGMENTS

We thank the Bioservices Group at Moredun Research Institute for their ongoing help and expertise and United Kingdom farmers for allowing access to sites for *D. gallinae* collection. We thank Alex Wilson (University of Miami) for helpful discussion during the course of this project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.695346/full#supplementary-material

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frontiers in Microbiology

REVIEW
published: 17 March 2022
doi: 10.3389/fmicb.2022.854803

The Symbiotic Continuum Within Ticks: Opportunities for Disease Control

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OPEN ACCESS

Edited by:
Olivier Duron, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Jianmin Zhong, Humboldt State University, United States
Jingwen Wang, Yale University, United States

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Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *14 January 2022*
Accepted: *15 February 2022*
Published: *17 March 2022*

Citation:
Hussain S, Perveen N, Hussain A, Song B, Aziz MU, Zeb J, Li J, George D, Cabezas-Cruz A and Sparagano O (2022) The Symbiotic Continuum Within Ticks: Opportunities for Disease Control. Front. Microbiol. 13:854803. doi: 10.3389/fmicb.2022.854803

Among blood-sucking arthropods, ticks are recognized as being of prime global importance because of their role as vectors of pathogens affecting human and animal health. Ticks carry a variety of pathogenic, commensal, and symbiotic microorganisms. For the latter, studies are available concerning the detection of endosymbionts, but their role in the physiology and ecology of ticks remains largely unexplored. This review paper focuses on tick endosymbionts of the genera *Coxiella*, *Rickettsia*, *Francisella*, *Midichloria*, and *Wolbachia*, and their impact on ticks and tick-pathogen interactions that drive disease risk. Tick endosymbionts can affect tick physiology by influencing nutritional adaptation, fitness, and immunity. Further, symbionts may influence disease ecology, as they interact with tick-borne pathogens and can facilitate or compete with pathogen development within the vector tissues. Rickettsial symbionts are frequently found in ticks of the genera of *Ixodes, Amblyomma*, and *Dermacentor* with relatively lower occurrence in *Rhipicephalus, Haemaphysalis*, and *Hyalomma* ticks, while *Coxiella*-like endosymbionts (CLEs) were reported infecting almost all tick species tested. *Francisella*-like endosymbionts (FLEs) have been identified in tick genera such as *Dermacentor, Amblyomma, Ornithodoros, Ixodes,* and *Hyalomma*, whereas *Wolbachia* sp. has been detected in *Ixodes, Amblyomma*, *Hyalomma*, and *Rhipicephalus* tick genera. Notably, CLEs and FLEs are obligate endosymbionts essential for tick survival and development through the life cycle. American dog ticks showed greater motility when infected with *Rickettsia*, indirectly influencing infection risk, providing evidence of a relationship between tick endosymbionts and tick-vectored pathogens. The widespread occurrence of endosymbionts across the tick phylogeny and evidence of their functional roles in ticks and interference with tick-borne pathogens suggests a significant contribution to tick evolution and/or vector competence. We currently

understand relatively little on how these endosymbionts influence tick parasitism, vector capacity, pathogen transmission and colonization, and ultimately on how they influence tick-borne disease dynamics. Filling this knowledge gap represents a major challenge for future research.

Keywords: tick microbiota, tick symbiont, symbiont-pathogen interactions, tick physiology, tick-borne diseases ecology, tick continuum

INTRODUCTION

Ticks are hematophagous, obligate ectoparasites of terrestrial vertebrates such as amphibians, reptiles, birds, mammals, and humans (Black and Piesman, 1994; Estrada-Peña and Jongejan, 1999; Stafford et al., 2007; Anderson and Magnarelli, 2008). Ticks play a significant role in transmitting infectious diseases (Hussain et al., 2021b; Perveen et al., 2021b), and are competent vectors of a wide range of pathogens affecting animal and human health globally (de la Fuente et al., 2017; Hussain et al., 2021a,c). They are the most important vectors among those transmitting vector-borne pathogens to animals, and the second, after mosquitoes, for pathogens with impact for human health (de la Fuente et al., 2017; Hussain et al., 2021a,c). As a taxonomic group, ticks are well-adapted to a wide range of climatic conditions, thriving in tropical, temperate and even subarctic habitats (Anderson and Magnarelli, 2008). Ticks (Acari: Ixodida) are divided into three families: Ixodidae, Argasidae, and Nuttalliellidea (Anderson and Magnarelli, 2008; Perveen, 2021), and almost 28 tick species have been reported to transmit pathogens to humans (Anderson and Magnarelli, 2008). The prevalence of many tick-borne pathogens, such as *Babesia*, *Theileria*, and *Borrelia* has increased in recent years due to climate change and other anthropogenic factors such as land use change, deforestation, urbanization, global travel, and trade (Rochlin and Toledo, 2020). Ticks acquire these pathogens during blood feeding on an infected vertebrate host (Raffel et al., 2014). Some colonize the tick midgut (Raffel et al., 2014), and migrate to the salivary gland from where they are transmitted *via* tick feeding to a new host. Although less common, direct transmission of some pathogens (e.g., *Borrelia afzelii*) from tick midguts has also been reported (Pospisilova et al., 2019).

Some bacteria, mainly present in the Malpighian tubules or ovaries of ticks, act as endosymbionts and are harmless to vertebrate hosts (Rowley et al., 2004). Obligate symbionts are indispensable for tick survival and fitness, frequently transmitted from adult females to their offspring, while some facultative symbionts as Cardinium or Spiroplasma are reproductive parasites and have major impacts on reproduction of arthropods (Perlman et al., 2006). Bacterial symbionts that are vertically transmitted from mother to offspring increase the fitness of the tick by directly enhancing the reproductive capacity of the vector, which in turn facilitates and increases the symbiont survival and persistence across arthropod hosts generations (Brouqui et al., 1993). However, whilst our current understanding of the life cycle of these symbionts is typically framed within vertical transmission only, horizontal transmission has been observed in a number of cases. For example, evidence of horizontal transmission has been reported in tick symbionts such as *Midichloria* (Bazzocchi et al., 2013), *Coxiella* (Shivaprasad et al., 2008; Seo et al., 2016; Mioni et al., 2020; Kobayashi et al., 2021) and *Arsenophonus* (Edouard et al., 2013) strains (Bonnet et al., 2017). Horizontal transmission is also common in symbionts of other arthropods such as parasitoid wasps (Parratt et al., 2016). Exposure of vertebrate hosts to tick symbionts have been regarded as evidence of the pathogenic potential of symbionts (Shivaprasad et al., 2008). Tick-host-symbiont relationships have been thus described as a continuum of "mutualism," "commensalism," or "parasitism" (Bonnet et al., 2017). But the role of vertebrate hosts in the ecology and life cycle of tick symbionts remains poorly understood due to a paucity of research in this area.

Other bacteria, that are neither human pathogens nor strict symbionts, can be regarded more generally as tick microbiota (Wu-Chuang et al., 2021a). Within the text, "microbiota" only refers to the microbes themselves, whereas "microbiome" refers to the microorganisms and their genes. Cowdry (1925) first recognized the relationship between ticks and their microbiome at the beginning of the twentieth century. His work reported *Rickettsia*-like bacteria in the ovaries, eggs, Malpighian tubules, and intestinal epithelial cells of 16 different tick species (Cowdry, 1925). The first tick microbiome study to employ next-generation sequencing (NGS) was published in 2011 by Andreotti et al. (2011). Since then, an increasing number of NGS studies have been used to characterize the tick microbiome, allowing for a broader view of its taxonomic composition in several tick species (Wu-Chuang et al., 2021a). In addition to pathogens and obligate endosymbionts, ticks carry commensal non-pathogenic microorganisms that complement tick nutrition and interact with tick-borne pathogens, affecting tick fitness and vector competence (Wu-Chuang et al., 2021a). Due to the complex nature of colonization of tick microbiota, the interaction between endosymbionts and pathogens is sometimes hard to understand, with these associations potentially affecting tick physiology and ecology (Bonnet et al., 2017). Nevertheless, it is known that changes in microbial communities can modulate vector competence by decreasing, for example, *Borrellia burgdorferi* colonization in *Ixodes scapularis* larvae (Narasimhan et al., 2014). Similar examples have been previously reviewed (de la Fuente et al., 2017; Wu-Chuang et al., 2021a). Not only the microbiota modulates pathogen colonization, but pathogen entry within the tick midgut milieu trigger changes in the microbial communities (Abraham et al., 2017). For example, *Anaplasma phagocytophilum* infection in ticks disturbs the gut microbiota, increasing the presence of *Pseudomonas* and reducing the abundance of *Rickettsia* and *Enterococcus* (Abraham et al., 2017), which was associated with a reduction of bacterial biofilms in

tick midguts (Heisig et al., 2014; Abraham et al., 2017) and also a decreased representation of biofilm synthesis pathways in the tick microbiome (Estrada-Peña et al., 2020b). *B. burgdorferi* infection increased the expression of the tick protein PIXR, which alter the gut microbiome, metabolome and immune responses and facilitates *B. burgdorferi* infection and molting of larvae (Narasimhan et al., 2017). Accordingly, it has been proposed that the relation between pathogens and microbiota is bidirectional (Cabezas-Cruz et al., 2018).

Developing a comprehensive understanding of the role of microbiota in governing the physiology and ecology of ticks, and its interaction with tick-borne pathogens could prove highly beneficial for devising new strategies to control and prevent tick-borne diseases. This review paper describes and discusses the most common symbiotic and endosymbiotic tick-microbiota relationships, highlighting their relevance for tick physiology and ecology. We focus primarily on published studies providing species-level resolutions for symbiont characterization, most often through PCR and whole genome sequencing. We also included studies using metagenomics approaches and NGS, a technique that has revolutionized microbiome research (Kulski, 2016), allowing for high throughput and high-resolution assessment of the assortment of circulating microorganisms in tick vectors. Thanks to these advances, it is now relatively straight-forward to characterize tick endosymbiont assemblages to species levels, with the resulting growing body of published work in this area prompting this review.

THE SYMBIOTIC CONTINUUM IN TICKS

Non-pathogenic microbes found in ticks can classified as commensals and endosymbionts. Within commensals, a highly variable group of bacterial taxa, referred to as microbiota, have been described (Narasimhan and Fikrig, 2015). The microbiota composition is under the influence of several factors including the tick species, physiological stress by environmental traits, blood-meal, host species, tick immunity, and developmental stage (Narasimhan and Fikrig, 2015). Despite the variability of bacterial taxa associated to ticks, bacterial communities in the tick microbiome are functionally redundant (Estrada-Peña et al., 2020a). For example, the microbiome of *I. scapularis* larvae and nymphs shared 80 taxa (24.6%, total 324), while out of 342 predicted metabolic pathways, 82.7%, were shared by all the tick samples (Estrada-Peña et al., 2020a). Furthermore, the *I. scapularis* microbiota exposed to pathogen infection, antimicrobial peptide or anti-tick host immunity shared a very reduced taxonomic core of 61 bacterial genera (7.4%, total 821), while the majority (i.e., 381) of the metabolic pathways (87.2%, total 437) were identified in all the samples (Estrada-Peña et al., 2020b), Also, despite high temperature altered the structure of the microbial community in *I. scapularis* (Thapa et al., 2019; Wu-Chuang et al., 2021b) four keystone taxa found across the temperature gradient, and their directly connected neighbors, contributed to more than 99% of the predicted pathways regardless the incubation temperature (Wu-Chuang et al., 2021b). This suggests that the tick microbiome is a stable source of metabolic functions with potential implications for tick physiology. A mechanism can then be proposed by which ticks are permissive to colonization by environmental bacteria fulfilling a specialized set of functions.

Intriguingly, metabolic pathways associated with nutritional complementation by symbionts are broadly distributed in the tick microbiome. For example, the presence of vitamin B synthesis genes in *Francisella* or *Coxiella* symbionts (see details below) compensate for the shortage of vitamin B in the blood meal (Duron et al., 2018). However, vitamin B synthesis genes are not restricted to symbiotic bacteria of the genera *Francisella* or *Coxiella*, but are widely distributed throughout several bacterial genera of tick microbiota (Obregón et al., 2019). In addition, considering the high diversity of genes and metabolic pathways encoded in the genomes of tick microbiota bacteria, we hypothesize that the contribution of bacteria to tick physiology and survival could extend well beyond vitamin B supplementation (Obregón et al., 2019; Estrada-Peña et al., 2020b). Indeed, the metabolic pathways associated with the tick microbiome include processes as diverse as amino acid, antibiotic, pyrimidine, lipid, and amino sugar metabolism (Obregón et al., 2019). Thus, an ecological-to-evolutionary continuum could be proposed in which environmental, free-living and/or host-associated, bacteria colonize tick tissues and later, under certain conditions, establish a symbiotic relationship with the tick host (**Figure 1**).

IMPACT OF SYMBIONTS ON TICK PHYSIOLOGY

The recent emphasis on tick microbiome research has provided a new dimension of the microbes carried by ticks. Scientists are investigating symbiotic interactions and their impact on invertebrate host's physiology (Guizzo et al., 2017). Ticks usually harbor two types of endosymbionts. The first group includes obligate mutualistic symbionts, transmitted from mother to offspring, which are essential for the development and growth of their host, supporting various functions such as nutrition (Wernegreen, 2012), and behavior (Zhong et al., 2021). The second group comprises facultative symbionts, which are not as important as their obligate counterparts in ensuring host survival (Ohtaka and Ishikawa, 1991). Nevertheless, facultative symbionts can also affect their host, where, for example, those found within reproductive organs can manipulate reproduction and physiology by inducing parthenogenesis and cytoplasmic incompatibility (Cordaux et al., 2011). *Coxiella*-like endosymbionts (CLEs) preferentially colonize the ovaries and Malpighian tubules of ticks (Cibichakravarthy et al., 2022). The colonization of CLEs in the ovaries of ticks promote reproduction and developmental processes and assists its maternal transmission to the offspring (Klyachko et al., 2007). The dense colonization of CLEs in Malpighian tubules assist in its nutritional role as Malpighian tubules are engaged in nitrogenous products excretion and osmoregulation (Dhooria, 2016; Cibichakravarthy et al., 2022). CLEs may recycle arthropods' hemolymph metabolites to synthesize

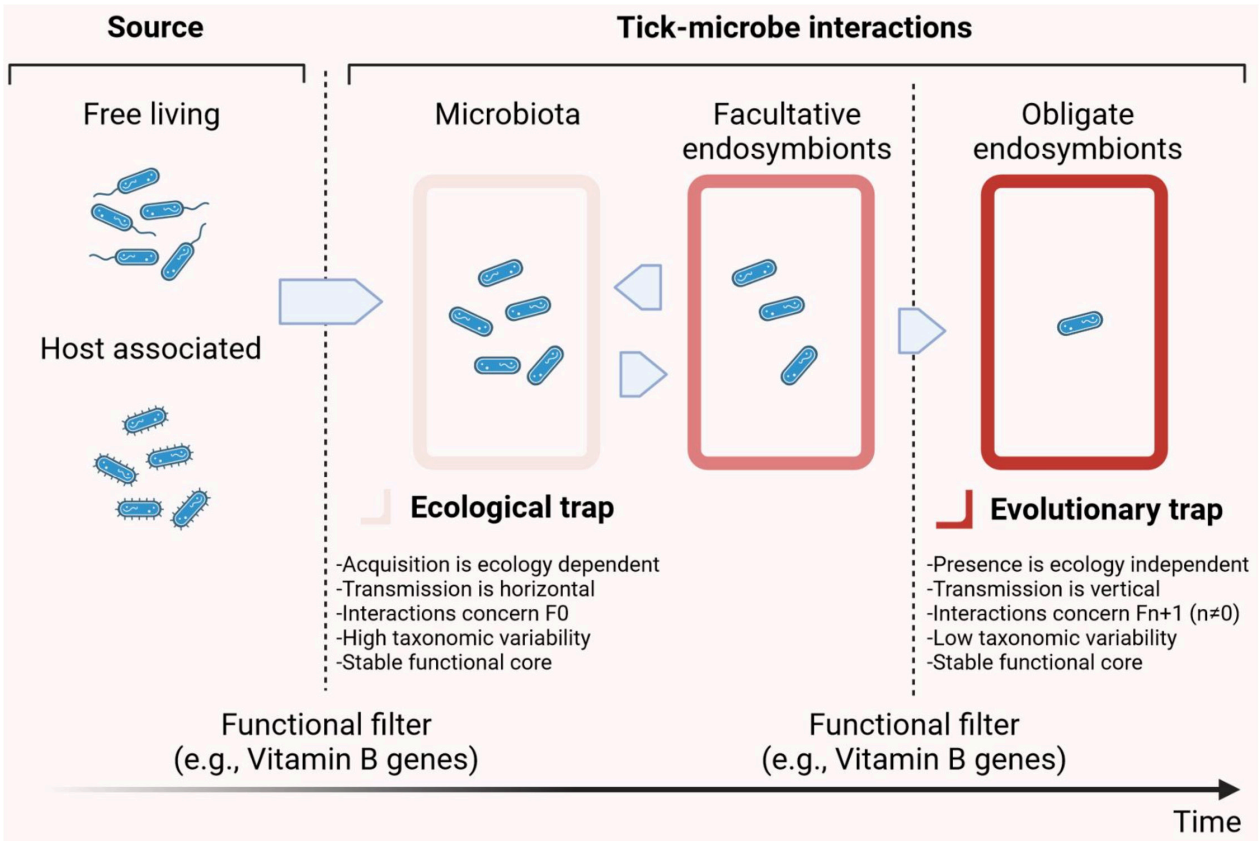

FIGURE 1 | Symbiotic continuum in ticks.

vitamin B (Cibichakravarthy et al., 2022). Further, vitamin B synthetic pathway enzymes have been detected significantly more abundant in Malpighian tubules as compared to ovaries (Cibichakravarthy et al., 2022) in *Rh. sanguineus*. FLEs are maternally inherited symbionts of ticks (Gerhart et al., 2016; Kumar et al., 2021). FLE was identified as an obligate nutritional mutualist in the life cycle of *Ornithodoros moubata* through experiments and synthesizes B vitamins (Duron et al., 2018) that are deficient in the ticks' blood meal. The elimination of *Francisella* F-Om alters ticks' life history traits (Duron et al., 2018). Comparison of the metabolic pathways present in FLE-Am to that of CLEAA showed that the metabolic capability of FLE-Am is extensive than CLEAA, for example, FLE-Am can produce heme in addition to cofactors (except thiamine) synthesized by CLEAA (Gerhart et al., 2016). Further, FLE provides cysteine, which found in fewer concentrations in blood meal and can synthesize glutamine from glutamic acid and ammonia, so recycling cellular ammonia waste to useful products. Therefore, FLE-Am has superior biosynthetic capability as compared to CLE (Gerhart et al., 2016). *Wolbachia* have been shown to influence the reproduction of infected insects in various ways, including parthenogenesis, male killing, cytoplasmic incompatibility, and feminization (Hurst et al., 1999; Stouthamer et al., 1999). *Candidatus* Midichloria mitochondrii is widespread in various ixodid ticks (Epis et al., 2008) was found abundant in unfed and semi-engorged *Ixodes ricinus* females that enhance the host fitness by supplying essential nutrients (Olivieri et al., 2019). In general, symbiotic partners enter into an evolutionary spiral that leads to an irreversible codependence with associated risks (Bennett and Moran, 2015), while the microbiota allows for more flexible tick-microbe interactions determined by and adapted to ecological conditions. Tick microbiota and its interactions with the tick and pathogens have been recently revised (Wu-Chuang et al., 2021a). In the sections below, we collate our current understanding about tick endosymbionts of the genera *Coxiella*, *Rickettsia*, *Francisella*, *Midichloria*, and *Wolbachia*, as summarized in **Supplementary Table 1**.

Coxiella

Coxiella-like endosymbionts is a obligate intracellular, maternally inherited bacterium, and found in high prevalence in tick populations (Duron, 2015). It usually engaged in mutualistic interactions with tick hosts (Brenner et al., 2021; Nardi et al., 2021). Several tick genera have been found to harbor CLE, including *Amblyomma* (Clay et al., 2008), *Dermacentor* (Jiao et al., 2021), *Haemaphysalis* (Lee et al., 2004), *Rhipicephalus* (Špitalská et al., 2018), *Ixodes* (Kurtti et al., 2002; Schabereiter-Gurtner et al., 2003; Špitalská et al., 2018), *Ornithodoros* (Duron, 2015), and *Carios* (Reeves et al., 2005). Douglas (2007) proposed that the benefits derived from the symbiotic relationship with

CLE is limited in ticks, but it is considered that they play an important role in the synthesis of several nutrients that are required by their tick hosts (Moran et al., 2003; Douglas, 2007). As strict hematophagous parasites, ticks rely on the nutritional composition of host blood to support their metabolism (Heisig et al., 2014). Nutritional deficiencies in the blood, such as the lack of sufficient amounts of vitamin B, provide the basis for symbiotic partnerships with bacteria that synthesize vitamin B. This is a common challenge for arthropods feeding on blood, which frequently carry bacteria that synthesize vitamins and thus contribute to the fitness of their tick host (Beard et al., 2001; Akman et al., 2002; Wu et al., 2006). Further, Zhong et al. (2021) demonstrated that reduction in the abundance of the CLE in *Haemaphysalis longicornis* (CHI) decreases blood intake in ticks. It was found that reduced CLE abundance reduces serotonin biosynthesis that is essential in regulating tick-feeding activity. Researcher determined that providing tetracycline-treated ticks with the CHI-derived tryptophan precursor chorismate restores the feeding defect. Further, its increased level in the synganglion and midgut promotes tick feeding. Herbicide glyphosate treatment suppresses blood-feeding behavior in ticks by inhibition of CHI chorismate biosynthesis. Therefore, -CHI regulate tick feeding activity (Zhong et al., 2021).

The genome size of CLE is smaller than that of *Coxiella burnetii,* an obligate intracellular bacterium that causes Q fever in humans and animals (Stein and Raoult, 1992), and they are deficient in certain proteins including *recN* gene product that involved in DNA repair along with that *Amblyomma* bacterium may also lack the DNA repair function provided by *recN*, hence it impairs DNA replication (Jasinskas et al., 2007). This evidence indicated that this gene loss in this bacterium is the same as *C. burnetii* but also that it has a reduced genome, a common feature of obligate endosymbionts of invertebrates (Moran and Wernegreen, 2000; Jasinskas et al., 2007).

Coxiella-like endosymbionts have evolved as endosymbionts, being found at greater loads in the *A. americanum* tick salivary glands (Klyachko et al., 2007), where presence has been reported as necessary for ensuring the survival of this tick species. CLE also play a key role in the reproductive fitness of female *A. americanum* ticks, where clearance of this bacterium by antibiotic treatment reduces egg hatchability, and increases the time to oviposition (Zhong et al., 2007). CLEs have also been identified through field collection in *Rh. turanicus*, where these were higher in females than males, further supporting sex-specific benefits, driven by vertical transmission of these endosymbionts that occurs in *Rh. turanicus* after CLE proliferation and colonization in ovaries (Lalzar et al., 2014). Additional studies have shown that CLE are more abundant in ovaries and Malpighian tubules, but less abundant in other organs as salivary glands (Noda et al., 1997; Lalzar et al., 2014; Wang et al., 2017; Buysse et al., 2019). As *Rh. turanicus* females display higher blood consumption and increased metabolic rates compared with males, sex-specific benefits could be expected to extend beyond reproduction in female ticks of this species, with CLEs suppling essential nutrients in larger quantities in feeding females. Indeed, research has shown a direct positive correlation between the abundance of symbionts and female weight during feeding in this species (Lalzar et al., 2014). In contrast, symbiont levels appeared to be lower in males than in females, where weight increases were also lower during feeding.

In the Cayenne tick, *Amblyomma cajennense*, CLE was identified in salivary glands, ovaries and Malpighian tubules (Machado-Ferreira et al., 2011). Further, this bacterium found with high prevalence rate (100%) in all life stages and in eggs of ticks that confirm its transovarial and transstadial transmission (Machado-Ferreira et al., 2011). Similarly, CLEs proteomes detected from Malpighian tubule (75%) and the ovaries (80%) of the brown dog tick, *Rh. sanguineus* (Cibichakravarthy et al., 2022)possible roles in metabolism, fecundity and osmoregulation (Machado-Ferreira et al., 2011; Sonenshine, 2014; Cibichakravarthy et al., 2022). It is reported that CLEs use few substances from the hemolymph for the synthesis of B vitamins (Magnúsdóttir et al., 2015), potentially explaining why they are rarely found in other tick organs, such as midgut and salivary glands. Nevertheless, the importance of tissue tropism of CLEs has not been fully elucidated (Duron et al., 2017).

Coxiella-like endosymbionts have also been found in *Haemaphysalis concinna* collected in different geographical areas of Russia (Mediannikov et al., 2003), and in *Rhipicephalus* in Switzerland (albeit in only a small fraction of the ticks sampled; Bernasconi et al., 2002), Such findings indicate that this symbiont group plays a variable role across different tick species, being important in many of those researched to date. Further investigation of CLEs in ticks could therefore be recommended to explain their role and importance in tick population fitness (**Supplementary Table 1**).

Rickettsia

Rickettsia is obligate, intracellular gram-negative bacteria (Perlman et al., 2006). These microbes are widely distributed in accordance with their tick hosts across the globe, causing many diseases in humans and animals. Despite being regularly reported as tick symbionts with ticks, however, the symbiotic potential of some species remains poorly understood (Parola and Raoult, 2001; Goddard, 2009). In cases where our understanding is clearer, these symbionts have been shown to play a vital role in tick physiology, fitness and population dynamics and pathogen transmission (Childs and Paddock, 2002). Endosymbiotic *Rickettsia* might have nutritional importance in ticks, as many phylotypes of *Rickettsia* can produce folate (vitamin B), which are not present in the blood of vertebrates on which ticks usually fed (Kagemann and Clay, 2013).

Through various studies, it has been shown that *Dermacentor variabilis* harboring symbiotic *Rickettsia* sp. demonstrate higher motility than ticks without *Rickettsia* sp. (Kagemann and Clay, 2013). *Rickettsia* sp. symbionts are most common in ticks of the genera of *Ixodes*, *Amblyomma*, and *Dermacentor*, whereas it has been less frequently found to in *Rhipicephalus*, *Haemaphysalis*, and *Hyalomma* ticks (Burgdorfer et al., 1973; Nováková and Šmajs, 2018). In *I. scapularis, Rickettsia* endosymbionts has been reported in 100% of eggs, 81% in larva, 90.5% in nymph, and 98.9% in adult females tested (Rounds et al., 2012). Identification of endosymbionts in ticks was done by broad-range polymerase chain reaction and electrospray ionization mass spectrometry.

The same study reports this bacterium to be present at high prevalence (100%) in *I. pacificus* (Rounds et al., 2012).

While *Rickettsia* symbionts may play roles in influencing the physiology of ticks, they often found as diverse species assemblages, interacting with one another. *Dermacentor andersoni* harbor symbionts such as *Rickettsia montanensis* (formerly *R. montana*) and *Rickettsia peacockii* (Burgdorfer et al., 1980; Macaluso et al., 2002), for example, and *D. variabilis* can be infected by *Rickettsia rickettsia, Rickettsia bellii*, and *R. montanensis* concurrently (Carmichael and Fuerst, 2006). It has been reported that *R. montanensis* is vertically transmitted in *D. variabilis*, but with this transmission inhibited by co-infection with a second *Rickettsia* sp. (Macaluso et al., 2002). Capillary feeding trials involving PCR diagnostics have also shown that ticks already harboring *Rickettsia* sp. are resistant to challenge with a secondary *Rickettsia* infection. In resisting co-infection, it has been proposed that *Rickettsia* present in tick ovaries change the molecular expression of oocytes, which prevents the occurrence of secondary residential infection (Carmichael and Fuerst, 2006). Soft ticks have also been reported to harbor *Rickettsia*. Particularly, two novel species named *Candidatus* Rickettsia Africa septentrionalis and *Ca.* Rickettsia mauretanica, were detected in *Ornithodoros occidentalis* from Morocco, *Ornithodoros erraticus* from Algeria and *Ornithodoros normandi* from Tunisia (Buysse and Duron, 2020).

Francisella

Francisella-like endosymbionts are gram-negative coccobacilli and facultative intracellular bacteria that are widespread in natural surroundings. In natural ecosystems, the survival of *Francisella* depends on temperature, direct sunlight exposure, and other physical factors (Kılıç, 2010; Celli and Zahrt, 2013). These bacteria are probably sustained in the environment through associations with various animals, including lagomorphs, rodents, insectivores, carnivores, ungulates, marsupials, birds, amphibians, and various species of invertebrates. Transmission to/between animals and humans may occur *via* bites of both ticks and mosquitoes (Momer, 1992; Eliasson et al., 2006). Ticks may serve as reservoirs, carrying the bacteria in their bodies throughout their lives, including in their salivary glands from where *Francisella* may be transmitted to new hosts at the site of tick feeding (Goethert and Telford, 2005; Gürcan, 2014; Yeni et al., 2021). *Francisella* have also been found in the reproductive tissues of female *D. andersoni* (Niebylski et al., 1997), with instances of unfed, infected larvae of *A. americanum* occurring in nature. Supporting the idea that transovarial transmission can occur (Calhoun and Alford, 1955). Transstadial transmission of *Francisella tularensis* has also been confirmed under laboratory conditions for multiple tick species, including *D. andersoni*, *D. variabilis* and *A. americanum* (Petersen et al., 2009).

FLEs are assumed to be non-pathogenic to humans, though they may cause limited pathogenicity in small animals (Keim et al., 2007) and are found in human-biting ticks, including those belonging to the genera *Dermacentor*, *Amblyomma*, *Ixodes*, and *Hyalomma* (Scoles, 2004; Machado-Ferreira et al., 2009; Ivanov et al., 2011; de Carvalho et al., 2016; Azagi et al., 2017). Presence of the genus *Francisella* has been reported in the camel tick, *Hyalomma dromedarii*, through 16s rRNA sequencing, with high relative abundance (99%) (Perveen et al., 2020b) and subsequent PCR confirming close relation to FLE (Perveen et al., 2021a). In the same geographic area, *H. dromedarii* ticks were reported throughout the year with high prevalence (94%) (Perveen et al., 2020a). It is possible that the high prevalence of camel ticks reported in this work may have been due to high abundance of these endosymbionts, though favorable microclimatic/environmental conditions and abundance of hosts for blood feeding (due to an increase in camel farming) may have also played a role. Previously, FLE was also detected with high prevalence in *Hyalomma* species (90.6%). In addition, maternal transmission rates of up to 91.8% were reported and FLE were localized in Malpighian tubules, ovaries, and salivary glands in *H. marginatum* (Azagi et al., 2017).

Francisella-like endosymbionts are closely related to pathogenic species of the genus *Francisella* (Noda et al., 1997; Scoles, 2004). Therefore, precise identification of this endosymbiont, its prevalence and interactions with other members of tick microbiota (co-existence) are crucial to understand and estimate its pathogenic potential, and investigate possible transmission to humans and animals. For example, the presence of closely related FLEs in tick species, including *D. variabilis*, *D. andersoni*, and *D. occidentalis* (Staples et al., 2006; Petersen et al., 2009) that can sustain and transmit pathogens causing tularemia poses a public health challenge.

Francisella-like endosymbiont is needed for tick growth and life cycle (Duron et al., 2018). In addition, FLEs have been found to be dominant symbionts in the Gulf Coast tick, *Amblyomma maculatum* microbiome (Binetruy et al., 2020) and reported in soft as well as hard ticks, especially in species belonging to the *Ornithodoros* genera (Gerhart et al., 2018). FLEs affect tick nutrition, and their capability to synthesize vitamin B make them especially suitable as mutualistic symbionts with *Ornithodoros moubata* (Duron et al., 2018). Further, elimination of FLE cause physical abnormalities and deficiencies in ticks that were restored by providing supplement of B vitamins (Duron et al., 2018). Gerhart et al. (2016) demonstrated the metabolic capability of FLE of *A. americanum* and found that FLE synthesizes cysteine, threonine, tyrosine, tryptophan, phenylalanine, and serine from pyruvate, and can break down glutamate, glutamine, and asparagine into ATP. This higher biosynthetic capability of FLE-Am could have led to replacing an ancestral symbiont, CLE in *A. maculatum* (Gerhart et al., 2016). Therefore, FLEs improve tick fitness by supplying vitamins and cofactors found in low concentrations in vertebrate blood (Gerhart et al., 2018).

Wolbachia

Wolbachia is an intracellular gram-negative bacterium that is the most commonly found microorganism infecting various arthropods. This bacterium is present in almost 60% of insect species and plays a role in multiple mechanisms, including altering host reproduction by inducing reproductive disorders, driving parthenogenesis, and acting as a defensive endosymbiont (Sazama et al., 2019). The presence of *Wolbachia* in ticks has been associated with parasitism by an *Ixodiphagus*

parasitoid (Plantard et al., 2012; Luu et al., 2021). *Ixodiphagus hookeri* (Hymenoptera, Chalcidoidea, and Encyrtidae) that are endoparasitoids of *I. ricinus*, were found infected with *Wolbachia pipientis* (99.2% prevalence) (Plantard et al., 2012). Further, it was reported that natural populations of *I. ricinus* ticks harboring *Wolbachia* were parasitized *by I. hookeri*, and ticks that were not parasitized by *I. hookeri* were *Wolbachia*-free (Plantard et al., 2012). Therefore, the occurrence of *W. pipientis* in *I. ricinus* ticks is probably attributable to the presence of *I. hookeri* (Plantard et al., 2012). Excluding the transmission of *Wolbachia* from *Ixodiphagus* to ticks, evidence suggests that *Wolbachia* is rarely found in ticks and other maternally inherited endosymbionts as *Spiroplasma*, *Midchloria*, or *Rickettsiella* are more common (Tijsse-Klasen et al., 2011). In agreement with this, this bacterium has been reported with a prevalence rate of only 14% in *I. ricinus* (Subramanian et al., 2012). Likewise, in *A. americanum* it was found with a prevalence rate of 3.5–25% in females (Zhang et al., 2011).

In a study in Southern Maryland (United States), *Wolbachia* infection was present only at low frequency in nymphs of *A. americanum*, and observed primarily in females and only rarely in males. This significant difference in male and female ticks could be due to male-killing effects of infection or a sampling bias. The impact of *Wolbachia* species on the reproductive potential of *A. americanum* is still unknown and warrants further attention to investigate any relationship (Benson et al., 2004). Furthermore, a more extensive range of tick species should be examined for *Wolbachia*, evaluating sex-specific infection rates and the role of this bacterium in host reproduction processes. Indeed, this bacterium may have a potential role in the biological control of ticks, as it does for various disease vectors and other arthropod pests (Ahantarig and Kittayapong, 2011).

Candidatus Midichloria Mitochondrii

Candidatus Midichloria mitochondrii is an intracellular bacterium belonging to the order Rickettsiales (Stavru et al., 2020), mostly reported in *I. ricinus* and this bacterium has vertical transmission in this tick (Sassera et al., 2006; Mariconti et al., 2012a). This alphaproteobacterium colonize mitochondria and was found residing in the mitochondrial intermembrane space through electron microscopy (Sassera et al., 2006; Stavru et al., 2020). It has been noted as the most prevalent endosymbionts in *I. ricinus*, having 100% (Olivieri et al., 2019), and 44% (Sassera et al., 2006) prevalence rates in females and males, respectively. *Candidatus* Midichloria mitochondrii was found to be abundant in ovaries of female *I. ricinus* (Olivieri et al., 2019) and vertically transmitted symbiont reported with 100% prevalence in females and immatures. Further, *Ca.* Midichloria mitochondrii can be found in gut, rostrum, tracheae, Malpighian tubules, and salivary glands of *I. ricinus* females (Olivieri et al., 2019). Results suggest subpopulations of *Ca.* Midichloria mitochondrii with different specializations due to tissue tropism. Further, *in silico* metabolic reconstruction indicate that *Ca.* Midichloria mitochondrii could enhance the host fitness, help in the anti-oxidative defense, maintain of homeostasis, water balance, and stable its population through vertical and horizontal transmission in the tick host (Olivieri et al., 2019). *In silico* metabolic reconstruction from the *Ca.* Midichloria mitochondrii genome showed that several genes involved in interaction with *I. ricinus* and complete biosynthesis pathways for B vitamins, especially B9 (folate) and B7 (biotin) suggest its nutrient-provisioning role. However, more experiments are required to better understand the mechanisms underlying this symbiotic interaction.

Mariconti et al. (2012a) and Perlman et al. (2006) showed that 47 patients exposed to bites of *Ixodes ricinus* ticks were seropositive to antigens of *Ca.* Midichloria mitochondrii. This suggests that *Ca.* Midichloria mitochondrii antigens secreted together with tick saliva could trigger the human immune response, or that ticks could transmit this endosymbiont to humans. The second scenario raises the possibility that *Ca.* Midichloria mitochondrii can then be acquired by a second tick feeding on the same host. Making this an example of an endosymbiont completing a tick-to-host-to-tick transmission route similar to that reported of tick-borne pathogens. Whether, in addition to the vertical transmission, such host-mediated transmission route plays any role on *Ca.* Midichloria mitochondrii life cycle remains unknown. Coincidentally, patients found seropositive with *Ca.* Midichloria mitochondrii were among 80 tick-exposed patients hospitalized due to clinical signs of Lyme disease, and 31 of them were also found seropositive for *B. burgdorferi* sensu lato (Mariconti et al., 2012a). Deducing a direct relation between *B. burgdorferi* and *Ca.* Midichloria mitochondrii colonization in *I. ricinus* from co-occurrence data in ticks is not justified, as this endosymbiont is present in nearly 100% of tick females, while *Borrelia* prevalence in ticks is highly variable. However, whether patient exposure to *Ca.* Midichloria mitochondrii favors, or not, *Borrelia* infection and Lyme disease is an interesting, but unanswered, question. A previous report reported that the growth of a pathogenic *R. parkeri* in *A. maculatum* along with endosymbionts (Budachetri et al., 2018). Further, Budachetri et al. (2018) found that *R. parkeri* interaction with tick symbionts, FLE and *Ca.* Midichloria mitochondrii (CMM) modulate host's defenses by up-regulating tick selenoproteins.

MUTUALISTIC "PATHOGENS"

TBPs considered as having a "mutualist" relation with the tick, but not symbiotic. Some TBPs have vertical and horizontal transmission (e.g., *Babesia*) (Young et al., 2019) and others only horizontal (*A. phagocytophilum* and *B. burgdorferi*) (Jaarsma et al., 2019; Kurokawa et al., 2020). During blood feeding, various microbes are acquired that can trigger an immunological response in ticks. The immune system of ticks includes various enzymes and proteins, with endosymbionts also known to interact with tick immune function. For instance, *B. burgdorferi* can be phagocytosed by tick hemocytes (Zchori-Fein and Bourtzis, 2011; Hussain et al., 2021a). For instance, *I. scapularis* transmits the Lyme disease spirochete, *B. burgdorferi* whereas the *D. variabilis* is unable to transmit the bacterium to vertebrate host (Johns et al., 2001). It's due to immune function of both tick species. In *I. scapularis*, some *Borrelia* spirochete

were found connected with hemocytes, while several spiral-shaped intact bacteria were free in the hemolymph, however, in *D. variabilis* intact spirochetes were very less. Further, *I. scapularis* tissues contained culturable bacteria unlike *D. variabilis* tissues (Johns et al., 2001). *In vitro*, it was demonstrated that spirochetes motility and survival not reduced when they were incubated with *I. scapularis* hemolymph plasma, however, more than 50% spirochetes became non-motile after incubation of spirochetes with *D. variabilis* hemolymph plasma. Furthermore, *I. scapularis* showed immunotolerance against *B. burgdorferi* and slow phagocytic response whereas, *D. variabilis* showed high immunocompetence and rapid phagocytic activity to clear pathogens (Johns et al., 2001). *A. phagocytophilum*, an intracellular bacteria transmitted by *I. scapularis*. Its transmission is restricted by a member of the 5.3 kDa family of antimicrobial peptides expressed in the salivary glands of tick (Liu et al., 2012). Studies have been conducted on *A. phagocytophilum* how it manipulate gene expression and activate signaling pathways (Cabezas-Cruz et al., 2017). For instance, infection of *A. phagocytophilum* stimulates the expression of antimicrobial peptides that is mediated by the activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Liu et al., 2012) to control bacterial load in tick salivary glands. Cabezas-Cruz et al. (2017) discussed about how tick-pathogen interactions increase the fitness in tick hosts. Pathogens manipulate tick protective responses to facilitate infection, however, preserve tick feeding and vector capacity to assure the survival of the pathogens as well as ticks (Cabezas-Cruz et al., 2017). *A. phagocytophilum* infections in *I. scapularis* showed higher tick fitness due to the induced expression of a tick antifreeze glycoprotein that increases their survival in the cold conditions (Neelakanta et al., 2010).

SYMBIONT-PATHOGEN INTERACTIONS

Ecological relationships among organisms are broad and diverse. The interactions that underpin them can range from beneficial to harmful along a continuum (Pérez-Brocal et al., 2011). Accurately defining symbiotic, mutualistic, or pathogenic interactions is challenging, especially where they may be influenced by various external factors such as climate change, land-use change, and invasion of parasites and hosts into new geographic ranges. Modern methodologies are helping researchers to better map this complex landscape of interactions, where molecular-based diagnostics have proven especially useful in confirming associations between microbes and various protozoa, other bacteria, fungi, and/or animals (Pérez-Brocal et al., 2011).

Multiple pathogens (Baneth, 2014) and symbiotic bacteria (Noda et al., 1997) may co-exist in tick midgut. But, many endosymbiotic microorganisms have established themselves in the ovaries of female ticks (Noda et al., 1997), and are often transmitted to eggs (and subsequently nymphs) from the mother. Examples can be found from *Coxiella*-, *Francisella*-, and *Rickettsia*-like endosymbionts, which all affect/improve the host fitness (Ahantarig et al., 2013), and defense against environmental stress (Bonnet et al., 2017). In some cases, these endosymbionts may infect humans (Ahantarig et al., 2013), with this potentially then affecting the tick-borne microbes occurrence and transmission (Bonnet et al., 2017; Bonnet and Pollet, 2021). Pre-existence of another symbiont can also influence the performance of a different species. For instance, the vertical transmission of a second *Rickettsia* species in the *D. variabilis* is inhibited by pre-existing Rickettsial infection in these ticks (De Sousa et al., 2001). For example, *R. peacockii* is found in Rocky Mountain wood ticks, *D. andersoni* and displays a close phylogenetic relationship to *Rickettsia rickettsii*, a bacteria of the spotted fever group (SFG), but many of the characteristics of rickettsial pathogens are missing (Burgdorfer et al., 1980). The major difference between pathogenic and non-pathogenic SFG *Rickettsia* is the ability to proliferate within macrophages distinguishable factor in pathogenic *Rickettsia*. In addition, pathogenic *Rickettsia* may be able to increase the endoplasmic reticulum protein folding capacity, while non-pathogenic *Rickettsia* do not show such capacity (Curto et al., 2019). Similarly, -FLEs- have been detected in a range of tick genera, such as *Amblyomma, Hyalomma, Ixodes,* and *Ornithodoros* (Sun et al., 2000; Scoles, 2004; Machado-Ferreira et al., 2009; Ivanov et al., 2011; de Carvalho et al., 2016; Azagi et al., 2017). The occurrence of closely related *Francisella* sp. in *Dermacentor*. species that can transmit tularemia (Elston and Apperson, 1977) suggests an important role for precise screening of *F. tularensis* in laboratories by PCR (Kugeler et al., 2005). Indeed, full assessment of the pathogenic potential of FLEs is crucial and has recently been undertaken for *Rickettsia* species (Felsheim et al., 2009). This work demonstrated that symbionts previously considered to be non-pathogenic (Raoult and Roux, 1997) may in fact be the opposite, as was true for *Rickettsia slovaca* and *Rickettsia helvetica* (Raoult and Roux, 1997). This supports that the presence, infection rate, and ecological/biological roles of bacterial endosymbionts need to be examined closely to reveal their contribution to tick-borne diseases epidemiology.

IMPACT OF SYMBIONTS ON DISEASE ECOLOGY

Vector-borne zoonotic diseases are a global health threat and involve humans, pathogens, vectors, and wildlife (Harrus and Baneth, 2005; Collinge and Ray, 2006). Novel disease emergence can be especially significant and may be facilitated through the anthropogenic spread of disease vectors and pathogens across the globe. Virulent lineages of pathogens can also be distributed globally through anthropogenic activities, such as the international trade of animals (Cunningham et al., 2017), deforestation, agricultural development, and the magnitude of human interaction in the disease ecosystem, with climate change and socio-economic and environmental factors also influencing the dynamics of pathogen populations (Paul et al., 2016). The regional pattern of risk of pathogen transmission and circulation in an area is very important to human health and influenced by several factors. Spatial distribution of tick-borne pathogens, microclimatic conditions, and host abundance may affect pathogen dynamics, as well as the survival of ticks

as potential vectors. Host species presence and abundance may impact pathogen spread (Estrada-Peña and de la Fuente, 2014), as may host and tick vector competence, as influenced by vector density and longevity (de la Fuente et al., 2017). Logically, any habitat considered fit for the tick-borne pathogen's circulation and spread must meet the prerequisite of housing ticks and their hosts for any risk to be realized (Pfäffle et al., 2013). High-throughput sequencing approaches have also emphasized the potential implications of the composition, diversity, and functional role of tick microbial fauna to public health (de la Fuente et al., 2017).

Symbionts may impact disease ecology in two ways. Firstly, through their impact on tick physiology they can contribute to increase fitness and vector abundance which indirectly increases pathogen circulation and disease risk. Secondly, symbionts compete or favor pathogen colonization and can directly impact disease ecology by decreasing or increasing the chance of host being bitten by tick able to transmit pathogens.

Symbionts may benefit ticks through impacts on their survival, growth, and defense systems (Ahantarig et al., 2013; Bonnet and Pollet, 2021), all of which can have knock-on effects to their significance as disease vectors for humans. The assessment of symbiont associations is very important because these impact tick reproduction and fitness (Ahantarig et al., 2013). For example, a decrease in progeny number and increase in oviposition time has been reported in *A. americanum* following the elimination of CLEs through antibiotics (Zhong et al., 2007). Other key processes in ticks, as supported through their symbionts, could potentially be targeted in a similar fashion. *Candidatus* Midichloria mitochondrii in *I. ricinus* affect tick molting process (Zchori-Fein and Bourtzis, 2011; de la Fuente et al., 2017). Further, recent sequencing and analysis of this bacterium genome suggest that the bacteria may serve as a source of ATP for the host cell during oogenesis (Epis et al., 2008; Gnainsky et al., 2021). *Rickettsia*-infected *Dermacentor* ticks showed more motility than uninfected ticks (Kagemann and Clay, 2013). Higher motility associated to host-seeking behavior in ticks indirectly influences infection risk by increasing the rates of tick bites and pathogen transmission. Therefore, for a given tick species, various symbionts and their population rate that varies across geographic locations (Lalzar et al., 2012; Duron et al., 2017) affect vector fitness, their abundance and impact disease risks.

Symbionts may interfere with pathogens replication and transmission by affecting pathogen diversity and abundance in various tick species and their transmission to humans and animals (Bonnet et al., 2017). Because symbionts compete with pathogens for nutrients or tissues and may excrete molecules directly inhibiting the growth of pathogens or facilitate pathogens development by immunosuppressing the invertebrate hosts (Bonnet et al., 2017). For example, *Wolbachia* may affect with the replication and transmission of pathogens such as bacteria, viruses, protozoa, etc. and protect arthropods from parasite-induced mortality, may be through up-regulating the immune system (Brownlie and Johnson, 2009). Further, *Wolbachia* can cause pathogen interference by reducing the chance of pathogen infection and decrease pathogen load, and cytoplasmic incompatibility by reducing hatchability of eggs (when infected males mate with uninfected females) in mosquitoes (Caragata et al., 2016). Several pathogens may affect vector competence (Hajdusek et al., 2013). Tick innate immunity involves several cellular and humoral response pathways that mediate defense to various infections caused by microorganisms, such as *Borrellia, Flavivirus,* and Babesia (Hajdusek et al., 2013; de la Fuente et al., 2017). Tick symbionts may influence tick immunity that in turn may influence pathogen infection. FLE in *D. andersoni* was found positively associated with pathogenic *Francisella novicida* infection (Gall et al., 2016). The presence of FLEs has positively influenced the establishment of *F. novicida* in *D. andersoni* that may suppress the tick immune system favoring the acquisition of *F. novicida* (Gall et al., 2016). Therefore, these microbial associations allow symbionts to facilitate or limit pathogen transmission and directly influence vector-borne infections.

USING OBLIGATE HEMATOPHAGY AND ENDOSYMBIONTS AS WEAK SPOTS FOR THE CONTROL OF TICKS AND TICK-BORNE PATHOGENS

As obligate hematophagous parasites, ticks ingest large amounts of blood from the vertebrate host during feeding. The tick midgut is the first organ in contact with host immune components present in the blood. After crossing the gut barrier (Ackerman et al., 1981; Ben-Yakir et al., 1987; Wang and Nuttall, 1994), host antibodies (Willadsen, 1997) and complement proteins (Rathinavelu et al., 2003) can reach the tick hemolymph (Ackerman et al., 1981; Ben-Yakir et al., 1987; Wang and Nuttall, 1994) and access the tick ovaries and eggs (Galay et al., 2018) as well as salivary glands and be secreted back to the host (Wang and Nuttall, 1994). For example, in *D. variabilis* and *I. scapularis* ticks, the crossing of host IgG from the midgut into the hemocoel occur during the later phases of engorgement (Vaughan et al., 2002). Notably, the immune functions of antibodies and complement are retained in the tick tissues (Ackerman et al., 1981; Ben-Yakir et al., 1987; Wang and Nuttall, 1994). Host antibodies interact not only with tissues and surface proteins (Willadsen, 1997), but can also be specifically transported inside the tick cells where they can interact with intracellular proteins (de la Fuente et al., 2011; Rodríguez-Mallon et al., 2012, 2015). Functional host antibodies have also been shown to interact with symbionts in *Rodnius prolixus* (Ben-Yakir, 1987), and *Glossina morsitans* (Nogge, 1978), as well as with bacterial microbiota in mosquitoes (Noden et al., 2011) and ticks (Mateos-Hernández et al., 2020, 2021).

Targeting vector microbiota with host antibodies is the rationale behind anti-microbiota vaccines for the control of vector arthropods such as ticks (Mateos-Hernández et al., 2020, 2021). Immunization with a tick microbiota Enterobacteriaceae, caused significant mortality of engorging ticks (Mateos-Hernández et al., 2020). Antibodies against the glycan α-Gal, present in tick bacterial microbiota, were associated with a mean mortality of approximately

45% in ticks fed on α1,3GT-deficient mice (Mateos-Hernández et al., 2020). Anti-microbiota vaccine directed at Enterobacteriaceae in the microbiota of *I. scapularis* disrupted both the makeup and functions of the microbiome and decreased pathways central to lysine degradation (Mateos-Hernandez et al., 2021). Anti-microbiota vaccines are a microbiome manipulation tool for the induction of infection-refractory states in the vector microbiome (Maitre et al., 2022).

The preliminary results with anti-microbiota vaccines justify their use to target tick endosymbionts for vector and/or tick-borne pathogen control. *Rhodnius prolixus* fed exclusively on blood from rabbits immunized against the symbiont *Rhodoccocus rhodnii* have developmental alterations such as prolonged molting times, incomplete development, and malformed limbs (Ben-Yakir, 1987). Feeding of *Rhodnius prolixus* larvae on hosts immunized against their symbiont produces retardation of the symbiont growth (Ben-Yakir, 1987). Developmental alterations observed in *R. prolixus* fed on *Rhodococcus rhodnii*-immunized animals were similar to those described in aposymbiotic triatomines (sterile raised and germ-free insects that lack *R. rhodnii*) (Salcedo-Porras et al., 2020). Similar results were obtained by Nogge (1978) who found that tsetse flies fed on rabbits immunized with symbionts became aposymbiotic and their fecundity decreased drastically while their longevity was not affected. Furthermore, *Glossina morsitans morsitans* flies maintained on rabbits immunized with gut bacteria had high mortality rates and permanently laterally extended wings, which in turn impairs flying and therefore trypanosomes transmission (Kaaya and Alemu, 1984). In addition to targeting tick endosymbionts with live-bacteria vaccines (Mateos-Hernández et al., 2020, 2021), selected enzymes encoded in the symbiont genome and transcriptionally active could also be targeted with host antibodies.

CONCLUSION AND PERSPECTIVES

In this review, we have discussed five main tick-borne endosymbiont groups, comprising *Coxiella-*, *Rickettsia-*, *Francisella-*, *Wolbachia*, and *Midichloria* -like species, reporting key symbiont-pathogen interactions and exploring potential impacts of symbionts and their associations on tick physiology and tick-borne disease ecology. Examples are provided of various bacteria that can benefit their host's health, with further examples of opportunistic or pathogenic bacteria that may stress the host in some way while exploiting it. It is clear from many of these examples that tick-symbiont relationships are complex and context-specific, and better defining the pathways that dictate how pathogenic microbes contribute to disease ecology will allow improved treatments that target the virulence responses associated with this microbiota.

Endosymbionts play a significant role in providing nutrients (Greay et al., 2018) that support host persistence, for example, rickettsial symbionts of *D. andersoni* and *Amblyomma americanum*, and the CLE of *A. Americanum*, and are essential for the fitness of their tick hosts (Greay et al., 2018). Experimentation using antibiotics have shown that cleansing ticks from its bacterial endosymbionts have major negative impact of vector fitness and survival as well as vector competence. For example, *D. andersoni* exposure to oxytetracycline caused a significant decline in tick feeding, molting competence, and survival (Wade et al., 2014). Similarly, injecting laboratory-reared *A. americanum* ticks with rifampin resulted in reduced endosymbionts (*Coxiella* sp. and *Rickettsia* sp.) and a reduction in weight of adult ticks post-molting (Zhong et al., 2007). However, antibiotic overuse is associated to several undesirable effects such as the emergence of microbial antibiotic resistance, agro-ecosystem contamination, and alteration on animal and human microbiota with potential negative effect on health. In addition, the effect of antibiotics on the microbiota is not specific, as several bacterial species can be depleted by antimicrobial treatment. All this, had led to the implementation of strong regulations to limit antibiotic use, particularly in livestock. Therefore, preventing the use of antibiotics to target tick endosymbionts. In this context, anti-microbiota vaccines emerge as an environmentally friendly alternative to target endosymbionts for the control of ticks and tick-borne pathogens.

The latest high throughput molecular diagnostic approaches have enabled researchers to explore microbiomes of different arthropods species rapidly and cost-effectively. However, the microbial composition of many tick species still needs to be explored, and additional studies are required to understand the functional role of these microorganisms in disease ecology and epidemiology (Greay et al., 2018). Tick-borne infections are a severe threat to public health and food security globally, and researchers, scientists, public health professionals and veterinarians must work both independently and collaboratively to reduce the risk they pose. Vector-borne infections thrive where infectious pathogens, competent vectors, and an infection-compatible microbiome exist, with disruption of any one of these drivers likely to reduce overall pathogen transmission by vectors (Maitre et al., 2022). Manipulation and targeting of tick endosymbionts have been under-explored to this end to date but may provide significant opportunities in the future.

AUTHOR CONTRIBUTIONS

SH conceived the study and searched the literature. SH and OS planned and designed the review manuscript. SH, NP, AC-C, and OS screened and organized the data. SH, NP, AH, AC-C, and OS drafted the manuscript. AC-C provided intellectual inputs and share ideas. SH, NP, AH, AC-C, OS, MA, BS, and JZ revised the manuscript. OS, AC-C, DG, and JL critically revised the manuscript. All authors read and approved the final manuscript.

FUNDING

OS is a Principal Investigator of an Internal Research Fund of the Department of Infectious Diseases and Public Health of the City University of Hong Kong (Project number 9380108).

ACKNOWLEDGMENTS

We are thankful to all authors whose articles are included in this study. We also appreciate the Run Run Shaw library facility provided by the City University of Hong Kong.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.854803/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Microbiology

MINI REVIEW
published: 09 June 2022
doi: 10.3389/fmicb.2022.905826

The Tsetse Metabolic Gambit: Living on Blood by Relying on Symbionts Demands Synchronization

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Tsetse flies have socioeconomic significance as the obligate vector of multiple *Trypanosoma* parasites, the causative agents of Human and Animal African Trypanosomiases. Like many animals subsisting on a limited diet, microbial symbiosis is key to supplementing nutrient deficiencies necessary for metabolic, reproductive, and immune functions. Extensive studies on the microbiota in parallel to tsetse biology have unraveled the many dependencies partners have for one another. But far less is known mechanistically on how products are swapped between partners and how these metabolic exchanges are regulated, especially to address changing physiological needs. More specifically, how do metabolites contributed by one partner get to the right place at the right time and in the right amounts to the other partner? Epigenetics is the study of molecules and mechanisms that regulate the inheritance, gene activity and expression of traits that are not due to DNA sequence alone. The roles that epigenetics provide as a mechanistic link between host phenotype, metabolism and microbiota (both in composition and activity) is relatively unknown and represents a frontier of exploration. Here, we take a closer look at blood feeding insects with emphasis on the tsetse fly, to specifically propose roles for microRNAs (miRNA) and DNA methylation, in maintaining insect-microbiota functional homeostasis. We provide empirical details to addressing these hypotheses and advancing these studies. Deciphering how microbiota and host activity are harmonized may foster multiple applications toward manipulating host health, including identifying novel targets for innovative vector control strategies to counter insidious pests such as tsetse.

Keywords: tsetse, *Wigglesworthia*, microbiota, insect, epigenetics

OPEN ACCESS

Edited by:
Takema Fukatsu, National Institute of Advanced Industrial Science and Technology (AIST), Japan

Reviewed by:
Aurélien Vigneron, Max Planck Institute for Chemical Ecology, Germany

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Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *27 March 2022*
Accepted: *16 May 2022*
Published: *09 June 2022*

Citation:
Lee MH, Medina Munoz M and Rio RVM (2022) The Tsetse Metabolic Gambit: Living on Blood by Relying on Symbionts Demands Synchronization. Front. Microbiol. 13:905826. doi: 10.3389/fmicb.2022.905826

TSETSE (DIPTERA: GLOSSINIDAE)

Tsetse flies are Dipterans belonging to the superfamily of exclusive blood feeders, Hippoboscoidea. Tsetse are exclusively grouped in the family Glossinidae, within the monophyletic genus *Glossina*, and are divided into four groups: morsitans, fusca, palpalis, and austeni (Krafsur, 2009). Tsetse flies are found only in sub-Saharan Africa with the different groups occupying distinct ecological terrains and blood meal preferences which

effects the medical and agricultural significance of different species (Solano et al., 2010). Tsetse flies undergo adenotrophic viviparity (Benoit et al., 2015) meaning that a single larva develops *in utero* each gonotrophic cycle (**Figure 1**). Maternal secretions provide nutrition and seed larva with microbiota (Ma and Denlinger, 1974) through modified female accessory glands known as milk glands.

TSETSE FLY MICROBIOTA

A key feature in the evolution of eukaryotes has been the spatial and temporal partitioning of biochemical processes for the purpose of regulation (Chomicki et al., 1808; Martin, 2010; Gabaldón and Pittis, 2015). This partitioning reaches even greater complexity with the presence of microbiota and the necessity to coordinate their physiology with host biology particularly if they also rely on vertical transmission for their persistence as this additionally entails coordination with host reproductive biology. Tsetse flies possess a relatively simple core microbiota consisting of three different bacterial species (*Wigglesworthia glossinidia*, *Sodalis glossinidius*, and *Wolbachia pipientis*) varying in their occurrence and ranging in their impact toward host biology from parasitism to mutualism. The obligate mutualist, *Wigglesworthia*, is a focal point of this review and will be further discussed below. A commensal *Sodalis* (Dale and Maudlin, 1999) is not known to impact tsetse fitness but has emerged as a bacterium of interest as a target for paratransgenesis and introducing a trypanosome refractory phenotype (De Vooght et al., 2018). Lastly, *W. pipientis* (supergroup A) may be harbored by tsetse typically within reproductive tissues (O'Neill et al., 1993; Cheng et al., 2000; Balmand et al., 2013) which may result in cytoplasmic incompatibility between mating of differentially infected individuals (Alam et al., 2011).

PARASITIC TRYPANOSOMES

Tsetse flies are the obligate vectors of most African Trypanosomes, *Trypanosoma* species, with an association dating back about 35 million years (Steverding, 2008). Trypanosome parasites are the causative agent of Human African Trypanosomiasis (HAT; *T. brucei rhodesiense* and *T. b. gambiensis*), a debilitating disease caused by the parasitic invasion of the central nervous system which is lethal if left untreated. The disease is endemic to 36 countries in sub-Saharan Africa. Animal African Trypanosomiasis (AAT; *T. b. brucei*, *T. vivax,* and *T. congolense*) is a wasting disease caused by trypanosome infections of domestic animals, contributing to food insecurity within impacted areas. Trypanosome infections of tsetse impose a reproductive burden on females (Hu et al., 2008) likely due to competition for resources with some of these provided by the microbiota (Michalkova et al., 2014; Rio et al., 2019).

THE OBLIGATE TSETSE MUTUALIST *Wigglesworthia glossinidia*

Both sexes of tsetse feed exclusively on vertebrate blood and consequently have epidemiological significance toward trypanosome transmission. The blood, although rich in amino acids and iron, is particularly poor in B vitamins (Douglas, 2017), which are essential for animals. The provisioning of multiple B vitamins by the obligate mutualist *W. glossinidia* has enabled the restricted feeding ecology of the tsetse fly. The *Wigglesworthia* symbiont is the most predominant member of the tsetse microbiota (Aksoy, 1995; Chen et al., 1999; Aksoy et al., 2014; Tsagmo Ngoune et al., 2019) and inhabits the cytosol of specialized tsetse epithelial cells known as bacteriocytes that collectively form a bacteriome attached to the anterior midgut (**Figure 1**). *Wigglesworthia* cells are large,

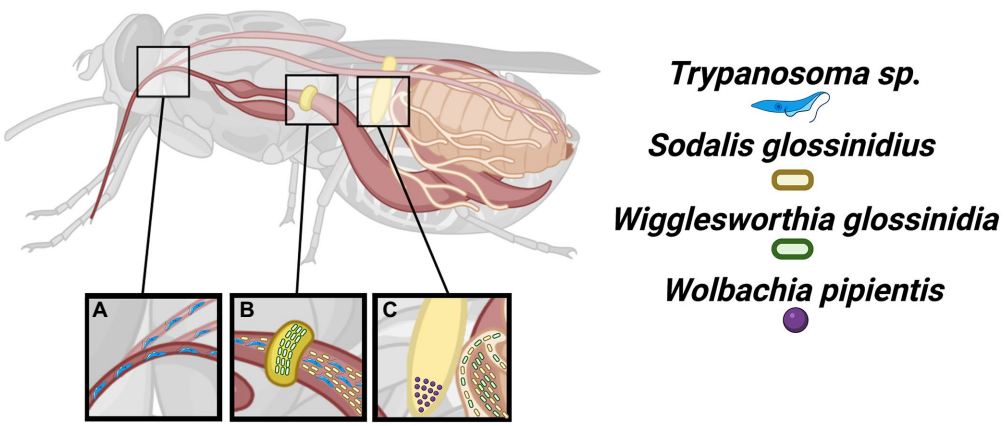

FIGURE 1 | Localization of tsetse microbiota. The tsetse fly is the sole vector of most African trypanosomes. **(A)** These protozoan parasites are introduced into the tsetse fly by an infected blood meal where developmental differentiation, recombination, and migration to the salivary glands occur. **(B)** The *Wigglesworthia* and *Sodalis* symbionts may be found within the bacteriome and gut, respectively. **(C)** The *Wigglesworthia*, *Sodalis*, and *Wolbachia* symbionts are vertically transmitted. The *Wigglesworthia* and *Sodalis* bacteria specifically use milk gland infections while *Wolbachia* infects ovaries for transgenerational persistence.

filamentous-like (Aksoy, 1995) and lie free in the cytoplasm, unabated from a host-generated membrane and likely also necessitating unique molecular transfer processes with tsetse.

The tsetse-*Wigglesworthia* association dates to around the incipient stages of species diversification of the Glossinidae family (Aksoy et al., 1995; Chen et al., 1999; Symula et al., 2011). This long interdependence has led to a profound impact on the evolutionary genomics of both species. Host adaptation has involved drastic *Wigglesworthia* genome size reduction (Akman et al., 2002; Rio et al., 2012) tailored to tsetse biology coupled with high genetic drift due to smaller population sizes arising from bottlenecks during vertical transfer. Despite its small size, the *Wigglesworthia* genome retains the potential to synthesize multiple B complex vitamins, namely, thiamine (B_1), riboflavin (B_2), nicotinamide (B_3), pantothenic acid (B_5), pyridoxine (B_6), and folate (B_9; Akman et al., 2002; Rio et al., 2012) believed to complement metabolic deficiencies in the blood feeding ecology of tsetse. To date, *Wigglesworthia* has not been cultured but with the availability of annotated genomes (Akman et al., 2002; Rio et al., 2012) and the advent of innovative culture technologies (Lagier et al., 2018; Cross et al., 2019) this may ultimately be achieved opening up a wide array of research questions. Additionally, the availability of an extracellular population of *Wigglesworthia* within maternal milk gland secretions (Ma and Denlinger, 1974; Attardo et al., 2008; Balmand et al., 2013) may also facilitate culturing.

As obligate mutualists, tsetse rely on *Wigglesworthia* for the optimal performance of several physiological processes involved in nutrition, digestion, immunological maturation and reproduction (and likely the connection between these; Wang et al., 2009; Snyder et al., 2010; Weiss et al., 2011, 2013; Michalkova et al., 2014; Snyder and Rio, 2015). In support of its specialization, the bacteriome is enriched in fly gene transcripts that belong to the transmembrane category (Bing et al., 2017; Medina Munoz et al., 2017, 2021), which includes amino acid transporters and multivitamin transporters, likely facilitating nutrient exchange between tsetse and *Wigglesworthia*. In turn, *Wigglesworthia* transcripts are enriched for the metabolism of cofactors and vitamins, supporting a complementary nutrient synthesis role for uptake by host transporters. Structural and functional examination of transporters, and how these may be regulated by epigenetics will help elucidate mechanisms used for interspecies metabolic regulation, likely involving some type of feedback network based on metabolites crucial for homeostasis, although this remains speculative.

EPIGENETICS AS COORDINATORS OF SYMBIOSIS

Epigenetics controls gene expression and concomitant phenotype independent of gene sequence (Choudhuri, 2011), thereby enabling a relatively rapid adaptation independent of inheritance. Epigenetic mechanisms within insects include small RNA production (Asgari, 2013, 2015; Lucas and Raikhel, 2013; Zhang et al., 2014a; Lucas et al., 2015), histone post-translational modifications (Dickman et al., 2013; Glastad et al., 2015), chromatin remodeling (Rider et al., 2010; Riparbelli et al., 2012), and DNA methylation (Field et al., 2004). Epigenetics may be heritable but may also be erased and reestablished to address specific environmental cues (Waddington, 2012; Tammen et al., 2013; Bind et al., 2014; Deans and Maggert, 2015; Chatterjee et al., 2018; McCaw et al., 2020; Villagra and Frías-Lasserre, 2020). Our focus in this mini review will be specifically on the roles that microRNAs (miRNAs) and DNA methylation may have toward mediating the coordination of microbe-host interactions.

microRNAs, PARAMOUNT SMALL REGULATORY ELEMENTS

miRNAs are small (~22 nt) noncoding RNAs with a primary function in sequence-specific post-transcriptional gene regulation (Ibáñez-Ventoso et al., 2008; Fabian et al., 2010). Gene regulation (generally inhibitory) *via* miRNAs is highly conserved across eukaryotes (Bartel, 2018) with sequence conservation of seed regions (i.e., nucleotides present at positions 2–8 from the 5′ end) facilitating identification across often phylogenetically distant animals (Ligoxygakis et al., 2002; Marco et al., 2010). For example, more than 50% of the characterized *Caenorhabditis elegans* miRNAs are encoded in both human and *Drosophila* genomes (Ibáñez-Ventoso et al., 2008; Asgari, 2011). High conservation of miRNAs among Dipterans has also been observed in studies comparing mosquitoes to *Drosophila* (Lai et al., 2003; Li et al., 2009). Despite this conservation, miRNAs sharing high nucleotide identity may exhibit target variation in different species by undergoing "seed-shifting," where slight changes in the 5′ end of a miRNA alters the seed region (Wheeler et al., 2009; Marco et al., 2010; Berezikov, 2011), consequentially generating a variety of new mRNA targets. Seed-shifting partnered with duplication is the primary evolutionary force for creating new miRNAs (Bartel, 2009; Berezikov, 2011). While miRNAs are known to be a significant source of regulators of endogenous genes (Carthew and Sontheimer, 2009), their potential role in modulating microbial homeostasis and in preventing dysbiosis has been comparatively understudied.

miRNAs are typically encoded within intergenic regions, in non-coding transcripts, or in rare cases within the coding region of genes (Slack, 2006; Asgari, 2011, 2013). To date, most miRNAs are produced through the canonical pathway, though a rare subset (known as non-canonical microRNAs) do not follow this pathway (Bartel, 2004; Abdelfattah et al., 2014). Canonical miRNA generation begins with transcription by RNA polymerase II of long RNA sequences known as polyadenylated primary transcripts (pri-miRNAs) in the nucleus (Asgari, 2011, 2013). The Drosha-Pasha/DGCR8 complex, also known as the Microprocessor complex, processes and cleaves the stem-loops of the pri-miRNA to form the hairpin precursor miRNA (pre-miRNA; Han et al., 2009; Asgari, 2011, 2013). The ~70 nt pre-miRNA is then transported into the cytoplasm by Exportin 5 and its terminal loop cleaved by Dicer and the *loquacious* protein (mammalian TRBP) forming a ~22 nt miRNA:miRNA* duplex (Asgari, 2011, 2013; Nguyen et al.,

2015). Similar to *Drosophila melanogaster* (Tomari and Zamore, 2005), tsetse flies also encode two distinct Dicer proteins (Dicer-1 and Dicer-2) with Dicer-1 required for miRNA production (Lee et al., 2004). Argonaute (Ago) proteins then associate with the miRNA duplex using one of the strands as a guide strand forming a RNA-induced silencing complex (RISC; Kawamata and Tomari, 2010; Nguyen et al., 2015). The remaining strand, miRNA*, is known as the passenger miRNA and may play a regulatory role but is typically degraded (Asgari, 2011, 2013). Dicer cleavage also seems to selectively favor an arm of the precursor stem loop, though this preference can vary in different tissues in a context dependent manner (Griffiths-Jones et al., 2011; Chen et al., 2018; Kim et al., 2020). This variation leads to 3′ or 5′ (typical represented as miRNA -3p or -5p) isomiRs being present for miRNAs (Kim et al., 2020). This arm shifting is also responsible for generating a large portion of the diversity within miRNA families (Okamura et al., 2008; de Wit et al., 2009; Berezikov, 2011; Griffiths-Jones et al., 2011).

It is likely that miRNAs have a diversity of functions within the tsetse fly, as within the related *Drosophila* species a range of roles in development, endocrinology, viral immunity, and behavior have been described (Carthew et al., 2017). This is further supported by the conservation of many miRNAs homologs in the more distantly related mosquitos, although miRNA conservation does not necessarily suggest functional retention since some miRNAs are also predicted to have numerous targets (Lai et al., 2003; Friedman et al., 2009; Li et al., 2009). Of relevance, in *Anopheles gambiae* mosquitoes, an elevated abundance of miR-305 is known to increase susceptibility toward *Plasmodium* infections (Dennison et al., 2015), likely mediated by disrupting mRNAs involved in metabolic (Lampe and Levashina, 2018) and immunological processes (Dennison et al., 2015). Similarly, shed trypanosome VSG surface coat antigen when internalized by tsetse cardia cells, decreases miR-275 expression within the midgut (Aksoy et al., 2016; Vigneron et al., 2018). Consequently, the reduction of miR-275 results in compromising the synthesis of the peritrophic matrix (PM) by inhibiting peritrophin expression, the Wnt-signaling pathway and Iroquois/IRX family of transcription factors in the cardia thereby disrupting digestion and strengthening vector competence (Aksoy et al., 2016). As a proof of principle, paratransgenic *S. glossinidius* engineered to express tandem antagomir-275 repeats (3xant-*miR275*) phenocopies the compromised peritrophic matrix and offers an exciting (and economical) technological advancement toward studying the regulatory roles of other miRNAs. Lastly, tsetse with symptomatic Salivary Gland Hypertrophy Virus (SGHV) infections exhibit different tsetse miRNA and SGHV miRNA expression profiles upon comparison to asymptomatic flies. With symptomatic flies, the most highly expressed miRNAs are predicted to target immune-related mRNAs, including those encoded by fibrillin-1 (*FBN1*) and Ras-related protein-27 (*Rab27*), and others involved in reproduction such as apolipoprotein lipid transfer particle (*Apoltp*) and vitellogenin receptor (*Vtgr*; Meki et al., 2018). These genes are all downregulated within symptomatic flies contributing to viral immune evasion and associated ovarian aberrations and loss of reproductive fitness (Abd-Alla et al., 2010).

In previous insect research low or absent miRNA homology suggests novel biological or physiological functions of that miRNA (Marco et al., 2010). Using a custom pipeline of bioinformatic tools on publicly available tsetse Expressed Sequence Tags (ESTs), 10 miRNAs were found to be unique to tsetse flies with gmr-miR 619-5p and gmr-miR-2490-3p predicted to target genes impacted by trypanosome infection, including those encoding the thioester-containing protein (Tep-1) and heat shock protein 60A (Hsp60a; Yang et al., 2020), although experimental validation of molecular regulation remains to be shown.

miRNAs may also directly impact microbiota composition and activity (Ibáñez-Ventoso et al., 2008; Friedman et al., 2009). Besides pathogenic associations, miRNAs are also involved in the regulation of essential members of the microbiota. For example, with the symbiosis between aphids and their symbiont *Buchnera* (a similar ancient obligate nutritional mutualism to the tsetse-*Wigglesworthia* association; Douglas, 1998; Feng et al., 2019), 14 aphid-generated miRNAs are evolutionarily conserved among phylogenetically distant aphid species with significantly different expression of these within bacteriomes relative to symbiont-free tissue (Feng et al., 2018) strongly supporting roles in mediating symbiosis. Moreover, 84 mRNA targets with a predominant function in the principal functional role of the symbiosis, amino acid transport and metabolism (Feng et al., 2018), were identified as putative targets of these miRNAs. At least 10 of the 14 miRNAs have been identified to be of importance toward other host–microbe interaction studies (Skalsky et al., 2010; Jayachandran et al., 2013; Mehrabadi et al., 2013; Mayoral et al., 2014a; Zhang et al., 2014b; Jin et al., 2017; Qiang et al., 2017; Liu et al., 2019) suggesting a universal (and likely convergent) role in the regulation of symbioses. Compellingly, research in tsetse has indicated genes associated with both amino acid transport and metabolism (*Wigglesworthia* is auxotrophic for the majority of amino acids) have differential expression in aposymbiotic compared to wild-type flies, which may indicate a similar regulatory role toward these genes could be played by tsetse miRNAs (Medina Munoz et al., 2017).

A plethora of questions remain about whether animals can use miRNAs to impact gene expression in microbes. Previous research on miRNAs in insect microbial relations has focused on identifying miRNAs produced by the host and assumed to target host mRNAs involved in the symbiosis (Carthew et al., 2017; Feng et al., 2018, 2019). Encouraging research that suggests targeting of microbial (particularly bacteria) RNA may in fact be plausible comes from studies demonstrating miRNAs regulating mitochondrial mRNAs (Li et al., 2012; Duarte et al., 2014; Macgregor-Das and Das, 2018). Mitochondria, as remnants of an ancient Alphaproteobacterium endosymbiont rendered modern-day organelle, still retain a double membrane (Macgregor-Das and Das, 2018) similar to *Wigglesworthia* (Aksoy, 1995). If tsetse miRNAs interact with *Wigglesworthia* to coordinate gene expression, they are likely not alone. It is possible that other mutualists with significantly reduced genomes

such as *Wigglesworthia* may also rely on these small RNAs as opposed to proteins for gene regulation, representing a novel avenue for experimental exploration to further our understanding of intracellular signaling (Hansen and Degnan, 2014). Lastly, a further compelling and reciprocal research focus is whether small RNAs encoded by bacterial mutualists may manipulate host genes, which is not unknown of within Gammaproteobacteria. For example, intracellular *Salmonella* produce a miRNA-like Sal-1 processed by human AGO2 proteins which enhances intracellular *Salmonella* survival (Gu et al., 2017). Further the production of a *Wolbachia* small noncoding RNA, *W*snRNA-46A, enhances the transcription of *Aedes aegypti* Dynein heavy chain (*Dhc*) which facilitates *Wolbachia* association with microtubules enabling its transfer during mosquito oocyte or embryonic development (Mayoral et al., 2014b). Whether small noncoding RNAs produced by *Wigglesworthia* may impact tsetse metabolism or immunity remains to be seen.

DNA METHYLATION AS A REGULATORY CONDUIT BETWEEN MICROBIOTA AND HOST PHYSIOLOGY

DNA methylation is the addition of methyl (CH_3) groups to cytosine residues (5mC) typically within 5′-cytosine-phosphate-guanine-3′ (CpG) dinucleotides (Lyko, 2018). Across insect taxa, genome methylation exhibits a patchy distribution and differs relative to those of vertebrates in regards to general localization (Head, 2014). For example, DNA methylation is prevalent in the promoter regions (creating CpG islands) of vertebrate genomes, with modifications altering the interactions of transcription factors and histones *via* steric effects (Moore et al., 2013). Within insect genomes, DNA methylation is pervasive within gene bodies (Cingolani et al., 2013; Takayama et al., 2014; Jeong et al., 2018; Huang et al., 2019), where it is involved in alternative gene splicing and the creation of isoforms (Lyko et al., 2010; Bonasio et al., 2012; Terrapon et al., 2014). Although CpG methylation is also present within insect genomes, methylation is more prevalent in the CpA and CpT dinucleotide contexts (Takayama et al., 2014). For example, splice junctions are enriched for non-CpG methylation (Cingolani et al., 2013) in bees and different splice variants of the same gene are associated with diverse methylation patterns (Lyko et al., 2010).

DNA methylation is among the most amenable epigenetic modifications to identify given its relative ease in identification. For example, commercially available antibodies detect the presence of methylated nucleotides within genomic DNA (Kunert et al., 2003) and may be used to enrich for methylated DNA prior to high-throughput sequencing (Glastad et al., 2014). Moreover, bisulfite sequencing and subsequent mapping (Ku et al., 2011), enables the characterization of nucleotide methylation across a reference genome of interest, permitting the discovery of preferential motifs (Takayama et al., 2014; Panikar et al., 2015). The generation of reference DNA methylomes for a variety of insects through developmental stages with validated ties to phenotypes will greatly facilitate our understanding of epigenetic modifications toward insect biology and fuel future research endeavors.

THE ROLE OF FOLATE TOWARD DNA METHYLATION

Although vitamins are essential to physiology, animals lack the ability to synthesize these *de novo* and must either obtain these critical nutrients through diet and/or microbiota provisioning (Brecher and Wigglesworth, 1944; Douglas, 2017). Folate (B_9) is particularly deficient within blood (Brecher and Wigglesworth, 1944; Edwards et al., 1957; Pietrzik et al., 2010; Nikoh et al., 2014; Douglas, 2017; Duron et al., 2018), with symbiotic bacteria often provisioning this essential cofactor to strictly hematophagous animals (Duron and Gottlieb, 2020). A significant role for *Wigglesworthia* within their hosts is the production and provisioning of folate, which is critical for tsetse reproduction and larval development while also serving to enhance vector competence (Snyder and Rio, 2015; Rio et al., 2019).

Folate is necessary for DNA methylation because it is transformed into 5-methyltetrahydrofolate (5-methylTHF), needed for the formation of methionine from homocysteine (Crider et al., 2012). Once methionine has been synthesized, it is joined to ATP and converted into the universal methylation donor *S*-adenosyl methionine (SAM). SAM donates the methyl group during DNA methylation *via* the action of DNA methyltransferases (Crider et al., 2012; Shorter et al., 2015). Folate provisioning by *Wigglesworthia* may provide a means for connecting *Wigglesworthia* metabolism to tsetse genetic regulation *via* DNA methylation. In support of this connection, SAM abundance is significantly decreased in tsetse fly bacteriomes which have been cleared of their *Wigglesworthia* symbionts (Bing et al., 2017).

Due to the lack of DNMT-1 and -3 in the genome, tsetse has been predicted to lack DNA methylation (Bewick et al., 2017). However, due to its close evolutionary relation to *D. melanogaster* and the characterization of DNA methylation in the fruit fly genome (Takayama et al., 2014; Panikar et al., 2015; despite also lacking these DNMTs), we hypothesize the presence of methylation in the tsetse genomic DNA, particularly within *Wigglesworthia* harboring bacteriomes which may impact symbiosis activities. Symbiosis altering DNA methylation is not unprecedented in eukaryotes as previously reported in a wide array of organisms including plants (Vannier et al., 2015), anemones (Li et al., 2018), and mice (Warner et al., 1989; Yu et al., 2015), with concomitant changes in symbiosis phenotypes.

DISCUSSION

Metabolite provisioning is a fundamental role of host-associated microbiota, particularly of animals with limited diets such as the strictly blood feeding tsetse fly. The tsetse fly provides a valuable, and medically significant, model system to dissect

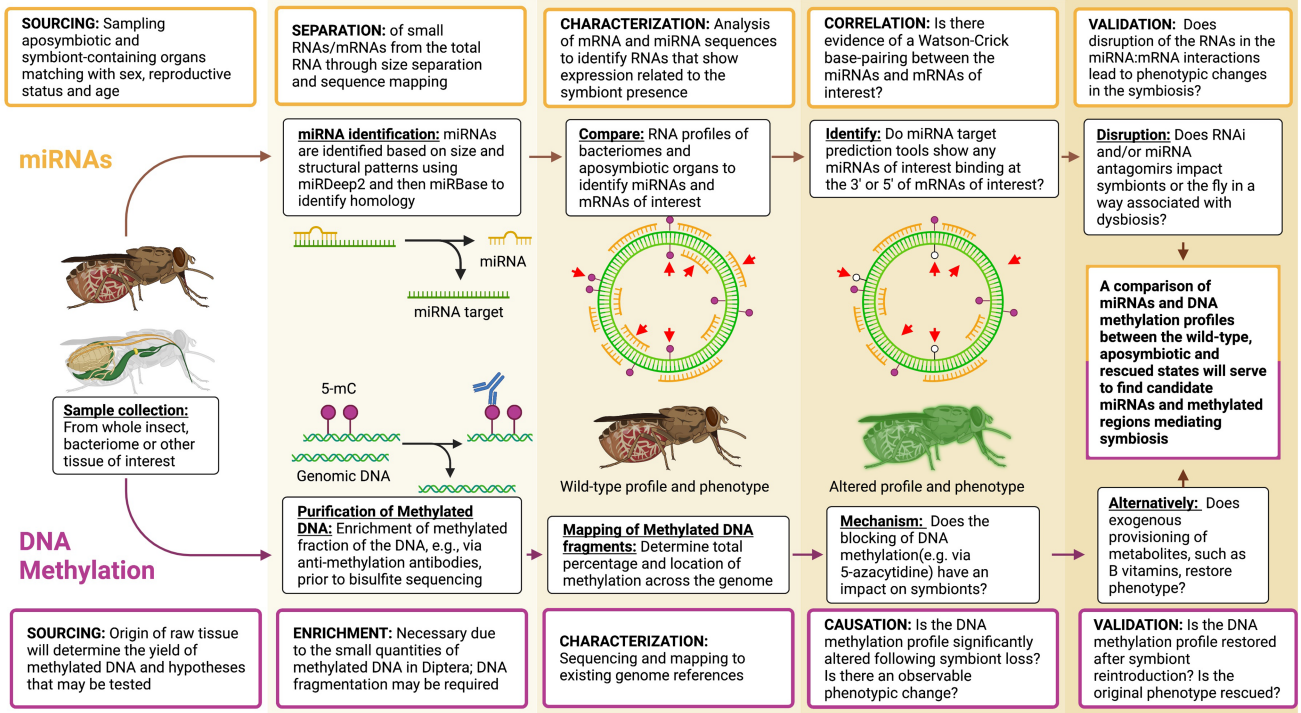

FIGURE 2 | Flow chart for the empirical analyses of epigenetic mechanisms mediating the *Wigglesworthia*-tsetse fly symbiosis. Of primary importance is the establishment of wild-type miRNA and methylation profiles and phenotypes followed by the characterization of altered states. If altered states are fully or partially rescued by restoration of the epigenetic mechanism through either the reintroduction of the symbionts and/or their provisioned metabolites, then there is evidence in favor of epigenetics mediating symbiosis. Distinct branches highlight methodology for the detection of miRNAs and DNA methylation impact toward symbiosis, while smaller boxes feature comments on the relevant methodology. Red arrows indicate differences in nucleic acid profiles upon comparison of sample groups.

regulatory mechanisms that coordinate host-microbiota activities, including nutrient exchange, immunological maturation and vector competence. Much has been gathered on the composition, functional contribution and evolutionary history of the tsetse microbiota, yet little is known regarding mechanisms coordinating microbial activity with host biology. Here we emphasize the investigation of epigenetics, specifically the role of miRNAs and DNA methylation, toward regulating interspecies activities as these may deliver rapid cues for the restoration and maintenance of homeostasis through tsetse development and following perturbations. We provide support for further investigations of these regulatory mechanisms and experimental guidance (**Figure 2**) for the simultaneous characterization of these epigenetic processes and assessing their impact toward the host-microbiota association. Besides providing the basis for a deeper understanding of ecological and organismal biology features and their evolution, the study of symbioses and its regulation, particularly in blood-feeding vectors is of significant consequence for epidemiological studies and the design of control strategies aimed at halting transmission of vector-borne diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

We acknowledge the support for writing this review by a WVU Eberly College Faculty Development Grant. The tsetse miRNA work done in our laboratory is supported by NIH-NIAID R21AI145271 (RR). Figures were created through BioRender.

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APPENDIX

Current research gaps

1. *Wigglesworthia*'s tight integration in tsetse physiology requires mechanisms for the regulatory control of population size and function. Intriguingly, *Wigglesworthia* lies free in the cytoplasm of bacteriocytes which may facilitate tsetse miRNAs to interact with these symbionts. Are tsetse miRNAs localized to the bacteriome acting on *Wigglesworthia* transcripts to control expression? Due to differences in nutrient demands during pregnancy and through aging, how may tsetse miRNA expression be impacted particularly toward the metabolic integration of *Wigglesworthia* symbionts?
2. *Salmonella* produce small RNAs which alter the host phenotype and lead to increased virulence. The DNA sequence which produces these small RNAs may also be found in many other Gamma-proteobacteria, making it plausible that these microRNA-like small RNAs have homologous roles in other bacteria. Do *Wigglesworthia* produce miRNA-like small RNAs to create favorable environments in the fly? Small bacterial RNAs represent an additional avenue for exploration toward advancing our understanding of interkingdom communication.
3. What types of epigenetic mechanisms may regulate the influx/efflux of substrates at symbiont and host transporters which lie at the interface of the association?
4. Pathogenic bacterial infections are associated with changes in the DNA methylation of several insects including members of Diptera (Ye et al., 2013; LePage et al., 2014), Lepidoptera (Baradaran et al., 2019), and Hemiptera (Negri et al., 2009). May beneficial symbionts also impact host DNA methylation? Establishing a cause-effect relationship and biochemical steps involved in these outcomes will be essential toward our understanding of the regulatory role exerted by bacteria on insect physiology.
5. What other epigenetic mechanisms may be affected by the metabolites of microbiota? For example, a role in epigenetics through biotin (B7) provisioning by symbionts may also occur given that histone biotinylation plays a role in transcriptional repression of genes and DNA repair.

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 01 August 2022
DOI 10.3389/fmicb.2022.900312

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

RECEIVED 20 March 2022
ACCEPTED 07 July 2022
PUBLISHED 01 August 2022

CITATION
Říhová J, Bell KC, Nováková E and Hypša V (2022) *Lightella neohaematopini*: A new lineage of highly reduced endosymbionts coevolving with chipmunk lice of the genus *Neohaematopinus*. *Front. Microbiol.* 13:900312. doi: 10.3389/fmicb.2022.900312

Lightella neohaematopini: A new lineage of highly reduced endosymbionts coevolving with chipmunk lice of the genus *Neohaematopinus*

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Sucking lice (Anoplura) are known to have established symbiotic associations multiple times with different groups of bacteria as diverse as Enterobacteriales, Legionellales, and Neisseriales. This diversity, together with absence of a common coevolving symbiont (such as *Buchnera*, in aphids), indicates that sucking lice underwent a series of symbiont acquisitions, losses, and replacements. To better understand evolution and significance of louse symbionts, genomic and phylogenetic data are needed from a broader taxonomic diversity of lice and their symbiotic bacteria. In this study, we extend the known spectrum of the louse symbionts with a new lineage associated with *Neohaematopinus pacificus*, a louse species that commonly parasitizes North American chipmunks. The recent coevolutionary analysis showed that rather than a single species, these lice form a cluster of unique phylogenetic lineages specific to separate chipmunk species (or group of closely related species). Using metagenomic assemblies, we show that the lice harbor a bacterium which mirrors their phylogeny and displays traits typical for obligate mutualists. Phylogenetic analyses place this bacterium within Enterobacteriaceae on a long branch related to another louse symbiont, "*Candidatus* Puchtella pedicinophila." We propose for this symbiotic lineage the name "*Candidatus* Lightella neohaematopini." Based on the reconstruction of metabolic pathways, we suggest that like other louse symbionts, *L. neohaematopini* provides its host with at least some B vitamins. In addition, several samples harbored another symbiotic bacterium phylogenetically affiliated with the Neisseriales-related symbionts described

previously from the lice *Polyplax serrata* and *Hoplopleura acanthopus*. Characterizing these bacteria further extend the known diversity of the symbiotic associations in lice and show unique complexity and dynamics of the system.

KEYWORDS

genome evolution, insect symbionts, lice, symbiosis, coevolution, *Neohaematopinus pacificus*

Introduction

Establishment of an obligate mutualistic symbiosis with nutrient-providing bacteria is an important evolutionary event that allows some insect groups to exploit new ecological niches (Sudakaran et al., 2017). Due to mutual dependence, such host-symbiont systems often undergo long-term coevolution manifested by mutually mirrored phylogenies between the host and the symbiont (Moran et al., 1993; Chen et al., 1999; Sauer et al., 2000; Dhami et al., 2013). This type of essential nutritional mutualist is usually called a primary symbiont (P-symbiont) characterized by several typical features, such as genome reduction, low GC content, and genomic deterioration of metabolic capacities (Toft and Andersson, 2010). In several insect groups, particularly those feeding on plant xylem or phloem sap, coevolution of the symbiont with the host can reach hundreds of millions of years into the past (Moran et al., 1993; Spaulding and von Dohlen, 1998; Sauer et al., 2000). In contrast, secondary symbionts (S-symbionts) are symbiotic bacteria with diverse phenotypes, not essential for their hosts (Stouthamer et al., 1999; Zchori-Fein and Perlman, 2004; Moran et al., 2005; Toh et al., 2006; Nováková et al., 2009), which are usually acquired more recently and may accompany P-symbionts in a particular taxon, or even a population of the host (McCutcheon et al., 2019).

In sucking lice of the order Anoplura, several obligate symbiotic bacteria have been described, generally thought to provide the hosts with compounds missing in their diets (Hypsa and Krizek, 2007; Fukatsu et al., 2009; Boyd et al., 2014, 2016; Allen et al., 2016; Rihova et al., 2021). Although all extant Anoplura share similar lifestyles and diets, no common ancient P-symbiont has been maintained through all lineages. Instead, several groups of sucking lice have acquired their symbionts independently from bacterial taxa as diverse as Enterobacteriales (Fukatsu et al., 2009; Boyd et al., 2014, 2016), Legionellales (Rihova et al., 2017), and Neisseriales (Rihova et al., 2021). While from the deep phylogenetic perspective the symbionts of sucking lice seem to form a diverse assemblage of different bacteria (Allen et al., 2016), at the more recent phylogenetic level some symbiotic lineages show tendencies to coevolve with their host for a limited period, and display features common for P-symbionts. These are, for example, the *Puchtella* lineages described from closely related species of *Pedicinus* (Boyd et al., 2017) and the *Legionella polyplacis* known at least from two *Polyplax* host species, *P. serrata* and *P. spinulosa* (Hypsa and Krizek, 2007). Some other louse symbionts seem to have been acquired more recently, and their genomes appear to have undergone a transition from S-symbionts toward P-symbionts (Boyd et al., 2016; Rihova et al., 2021). This ambiguous mode of symbiosis, i.e., short periods of coevolution intermixed with frequent losses and acquisitions, raises a question on the significance of the symbionts for their louse hosts. While considered obligate mutualists, essential for the host survival and reproduction, the exact role of the louse symbionts is not clear. Seventy-year-old experimental evidence obtained for *Pediculus humanus* suggests that this louse may depend on provisioning of most B vitamins by its symbiont (Puchta, 1955). However, genomic comparison of the louse symbionts for which complete genomes are available shows considerable inconsistency in preservation of their metabolic pathways, including those for B vitamins (Rihova et al., 2021). To better understand the evolution and significance of louse-symbiont systems, much broader taxonomic diversity has to be examined using genomic and phylogenomic tools.

In this study, we extend the current list of the investigated louse-symbiont systems by taking advantage of the recently described codiversification between several chipmunk species (Rodentia: Sciuridae: *Tamias*) and their lice of the genus *Neohaematopinus* (Bell et al., 2021). These lice are primarily parasites of sciurid rodents in Asia and North and Central America, where one species, *N. pacificus*, was described from western North American chipmunks in the subgenus *Neotamias* (Bell et al., 2015, 2021). Coevolutionary analysis between the chipmunks and lice indicates that rather than a single polyxenic species, the lice classified within *N. pacificus* form a cluster of separated phylogenetic lineages each specific to a single chipmunk species or group of closely related species (Bell et al., 2021). Utilizing metagenomic data and phylogenetic background from this coevolutionary study, we identify a new putatively obligate and mutualistic symbiont of lice, show its coevolution with the louse hosts, and reconstruct its metabolic capacity related to its likely symbiotic role.

Materials and methods

Screening and assembly of chipmunk lice metagenomic data

Metagenomic data from 21 specimens of *Neohaematopinus* lice was downloaded from the NCBI SRA database (Sequence Read Archive; Leinonen et al., 2011; **Table 1**). Initially, we employed a comprehensive search for bacteria by classifying metagenomic reads from each sample using the GOTTCHA2-v.2.1.7 signature-based metagenomic taxonomic profiling tool (Freitas et al., 2015) implemented in KBase (Arkin et al., 2018) with database built from NCBI RefSeq Release 90. The full taxonomic output reports were summarized at the strain and genus level in R with package phyloseq (McMurdie and Holmes, 2013). All the bacterial strains with relative abundances above 0.1% in any sample were considered.

Assemblies of the metagenomic data were generated using SPAdes v.3.13.0 (Bankevich et al., 2012) with the option, meta. The CONCOCT v.1.1 software (Alneberg et al., 2014) implemented in Kbase (kb_concoct 1.3.4) was ran with default parameters, using Bowtie2 (Langmead and Salzberg, 2012) as a default read mapper, to organize the contigs into putative metagenome-assembled genomes (MAGs) based on their nucleotide composition and depth of coverage. Metagenome-assembled genomes were classified into bacterial taxa using GTDB-Tk (Chaumeil et al., 2020) against the Genome Taxonomy Database (GTDB) v.R06-RS202 (258,406 bacterial and archaeal genomes) based on average nucleotide identity with a reference genome, the genome's position in the reference tree, or relative evolutionary divergence and placement of the genome in the reference. The GOTTCHA read classification and GTDB-Tk classification of MAGs (**Supplementary Table 1**) both indicated the presence of a prospective obligate endosymbiont in majority of the samples. In the GOTTCHA classification (**Supplementary Table 1**), a number of reads were assigned to the taxa *Buchnera*, *Baumannia*, and *Evansia*. GTDB-Tk classification of MAGs revealed several Enterobacteriaceae, including *Puchtella* sp. str. PRUG, endosymbiont of the louse *Pedicinus baddi* (**Table 1**). Among the other assignments, a potentially interesting taxon in respect to endosymbiotic interactions, was the Neisseriaceae-related bacterium (see "Results and discussion" section).

Refining metagenome-assembled genomes and completing genome drafts

To refine Enterobacteriaceae and Neisseriaceae MAGs, i.e., to detect potentially missed contigs and to remove the incorrectly binned ones, we used the following procedure (scheme provided in **Supplementary Figure 1**). The Enterobacteriaceae MAGs were present in twelve louse metagenomic assemblies, in five of them by only few long contigs (**Table 1**). For these twelve meta-assemblies, we filtered all putative Enterobacteriaceae contigs based on their GC content and coverage. For each assembly, these values were inferred from the contigs present in its Enterobacteriaceae MAG. From these extended bins, we then selected subsets of all prokaryotic contigs based on the ORFs density as predicted in Geneious Prime v.2020.2.5 (Kearse et al., 2012). To confirm that this approach detected all contigs belonging to the Enterobacteriaceae symbiont, we performed an alternative BLAST-based screening. We prepared databases from all 21 MAGs and screened them by BLASTn search (set to default E-value 10.0 and one best hit) using all genes from *Puchtella* sp. str. PRUG (NZ_LKAS01000001.1 + NZ_LKAS01000002.1) and two close relatives *Wiglessworthia glossinidia* (BA000021.3; NC_016893.1) and *Blochmannia pensylvaticus* str. BPEN (NC_007292.1) as queries. In addition, we used annotated genes from the best draft (the sample SRR12483207; for annotation procedure see below) as queries to screen the remaining 20 meta-assemblies by BLASTx searches (Camacho et al., 2009) set to default E-value (10.0) and one best hit. For all the contigs retrieved by these approaches we checked their taxonomical origin by BLASTn search (set to default E-value 10.0 and three best hits) against the nucleotide (nt) database. The Neisseriaceae-related MAG was present in a single meta-assembly (SRR12483206) and was largely fragmented. To refine this MAG, we used the same approach as described above for the Enterobacteriaceae. To screen all other meta-assemblies for possible presence of this bacterium, we applied the BLAST-based approach with the genes of the two Neisseriaceae-related louse symbionts (CP046107 and WNLJ00000000) as queries.

For the putative taxonomic assignment of each gene in the least fragmented genome of the Enterobacteriaceae symbiont (L207) we employed BLASTx search against the nr database set to three best hits (**Supplementary Table 2**). The completeness of the L207 draft was assessed by BUSCO v.4 (Simao et al., 2015) and for the Enterobacteriaceae symbiont also by the size convergence (see "Results and discussion" section). All genome drafts were annotated by PROKKA (Seemann, 2014) and RAST (Aziz et al., 2008) and deposited in GenBank under the BioProject accession number PRJNA813538 for the twelve most complete assemblies of Enterobacteriaceae symbiont (L222, L469, L220, L207, L201, L221, L218, L208, L2017, L209, L214, and L204) and the two most complete assemblies of Neisseriaceae-related symbiont (N206 and N204). To detect candidate pseudogenes in the annotated L207 and N206 assemblies, we used bioinformatic tool Pseudofinder (Syberg-Olsen et al., 2021).

10.3389/fmicb.2022.900312

TABLE 1 Overview of metagenomic binning and taxonomy assignment.

SRR data code	Number of paired reads	Read length (nt)	Bin count	GTDB classified MAG bins	Assigned taxonomy: family; genus	Bin total length (nt) and contig count
SRR5088469	154952566	100	18	bin.004.fa	Enterobacteriaceae	458733 (5)
				bin.006.fa	Burkholderiaceae; *Variovorax*	1723338 (455)
				bin.009.fa	Burkholderiaceae; *Burkholderia*	7009440 (797)
				bin.015.fa	Hyphomicrobiaceae; *Hyphomicrobium*	422320 (157)
				bin.016.fa	Rhizobiaceae; *Mesorhizobium*	9399685 (16661)
				bin.017.fa	Sphingomonadaceae; *Sphingomonas*	4635438 (84)
SRR12483222	69397816	160	116	bin.022.fa	Enterobacteriaceae	464809 (14)
SRR12483221	34725622	160	40	bin.015.fa	Enterobacteriaceae	459627 (5)
				bin.024.fa	Xanthobacteraceae; *Bradyrhizobium*	7557328 (558)
SRR12483220	32486852	160	121	bin.044.fa	Enterobacteriaceae	460455 (6)
SRR12483219	55847912	160	156	none	NA	NA
SRR12483218	80798234	160	105	bin.012.fa	Enterobacteriaceae; *Puchtella*	440570 (34)
SRR12483217	27859822	160	72	bin.059.fa	Lactobacillaceae; *Lactobacillus*	254633 (74)
				bin.061.fa	Enterobacteriaceae; *Puchtella*	378565 (47)
SRR12483215	23212912	160	104	none	NA	NA
SRR12483214	27929696	160	166	bin.058.fa	Enterobacteriaceae; *Wigglessworthia*	202321 (55)
SRR12483213	41335766	160	47	bin.014.fa	Lactobacillaceae; *Lactobacillus*	500811 (86)
				bin.041.fa	Xanthobacteraceae; *Bradyrhizobium*	6871847 (970)
SRR12483212	27333640	160	96	none	NA	NA
SRR12483211	21275976	160	105	none	NA	NA
SRR12483210	46206712	160	130	none	NA	NA
SRR12483209	29071184	160	136	bin.012.fa	Enterobacteriaceae; *Puchtella*	317573 (72)
SRR12483208	38144898	160	107	bin.072.fa	Enterobacteriaceae; *Puchtella*	462982 (6)
SRR12483207	27742464	160	10	bin.019.fa	Enterobacteriaceae	449562 (3)
SRR12483206	38930820	160	60	bin.014.fa	Xanthobacteraceae; *Bradyrhizobium*	2938854 (759)
				bin.043.fa	Neisseriaceae	1148191 (218)
SRR12483204	71767598	160	78	bin.015.fa	Enterobacteriaceae	262 958 (53)
SRR12483203	20092028	160	43	none	NA	NA
SRR12483202	60251848	160	118	none	NA	NA
SRR12483201	34764372	160	92	bin.087.fa	Enterobacteriaceae	465231 (6)

Phylogenetic analyses

Phylogenetic position of the new Enterobacteriaceae symbiont

Because preliminary analyses suggested phylogenetic position of the dominant symbiont within Enterobacteriales, we downloaded a representative set of available proteomes for each major lineage of Enterobacteriales from NCBI and JGI databases (proteome accession numbers provided in **Supplementary Table 3A**) including the putatively closest relatives indicated by the binning process and BLASTp against NCBI database (*Puchtella* and *Wigglessworthia*). Several gammaproteobacteria representing other orders were used as outgroups: *Vibrio cholerae* O1 biovar eltor str. N16961 (Vibrionales), *Pseudomonas aeruginosa* PAO1 (Pseudomonadales), *Candidatus* Evansia muelleri CEM1.1 (Oceanospirillales), and *Xanthomonas citri* pv. *vignicola* strain CFBP7111 (Xanthomonadales). BLASTp searches with default parameters (E-value 10.0) and *Salmonella enterica* (accession NZ_CP065718) protein queries for 14 orthologs from Husnik et al. (2011) were used to retrieve sufficiently long and reliably aligned sequences (list of the orthologs provided in **Supplementary Table 3B**) from all the proteomes. For

the new Enterobacteriaceae symbiont, we included two of the least fragmented assemblies (L207 and L221), for which we identified the corresponding orthologs by BLASTp (with default E-value 10.0) and verified these by visual inspection and by the gene's annotations. The single-gene amino-acid matrices were aligned by MAFFT v.7.450 (Katoh et al., 2002) using the E-INS-i setting implemented in Geneious Prime v.2020.2.5 (Kearse et al., 2012), visually inspected, and the genes concatenated. Ambiguously aligned regions were removed by Gblocks (Castresana, 2000) using the less stringent option. The concatenated matrix ("phylogenetic matrix") was analyzed using maximum likelihood (ML) and Bayesian inference (BI) for the phylogenetic reconstruction. The resulting matrix consisted of 7,438 amino acids. The ML tree was inferred using PhyML v.3.3 (Guindon et al., 2009) implemented in Geneious Prime with 100 bootstrap replicates and run under the best fitting CpREV + G + I evolutionary model selected by Akaike criterion (AIC) by smart model selection (SMS) algorithm (Lefort et al., 2017) implemented in the PhyML v.3.0 web server (Guindon et al., 2010). The BI analyses were performed using two different approaches. First, MrBayes v.3.2.6 (Ronquist et al., 2012) was run with four chains for 2,000,000 generations under the CpREV + G + I model. The chain convergence was evaluated in Tracer v.1.6 (Rambaut et al., 2018) and by the standard deviation of split (<0.01) and PSRF+ (reached the value 1.0). Since the *Neohaematopinus* symbionts formed a very long branch, placed within a cluster of other long-branched symbiotic bacteria, we also used PhyloBayes MPI v.1.8 (Lartillot et al., 2013) with CAT-GTR model to minimize possible artifacts due to the aberrant character of the sequences. The analysis was run for 50,000 generations and the quality of convergence was evaluated by the bpcom and tracecomp assessments. The list of bipartition differences indicated that while for most bipartitions the parameter dropped below 0.1, for two bipartitions it remained above 0.3. These problematic bipartitions included taxa distant from our focus, particularly the species of *Erwinia*. To improve the convergence and verify correctness of the topology, we prepared a "reduced phylogenetic matrix" containing 68 taxa (designated in **Supplementary Table 3A**) and retained the matrix of 7,438 amino acids. This matrix was analyzed in PhyloBayes with the same parameters (CAT-GTR, 50,000 generations), resulting in a drop of the maxdiff parameter to 0.057.

Host and the Enterobacteriaceae symbiont coevolution

To assess congruence between the host and the symbiont, we prepared host and Enterobacteriaceae symbiont matrices restricted to the datasets which provided sufficient data for the symbiont (12 host/symbiont samples sharing five orthologs; **Supplementary Table 3C**). Due to different characters and sizes of the matrices, the host and symbiont phylogenies were computed by different methods. The host phylogeny was reconstructed by two different methods. The first method employed ML analysis of 1,107 concatenated nuclear loci from Bell et al. (2021). The reconstruction was performed in IQ-TREE v2.0 (Minh et al., 2020) with the best model (GTR + F + R10) determined by ModelFinder (Kalyaanamoorthy et al., 2017) and support assessed by 1,000 ultrafast bootstrap replicates (Hoang et al., 2018), as implemented in IQ-TREE. In the second approach, we used IQ-TREE v2.0 to generate individual gene trees for the 1,107 nuclear loci and based on these trees, we then estimated a species tree in ASTRALIII v5.7.3 (Zhang et al., 2018), with local posterior probabilities (Sayyari and Mirarab, 2016). For the assemblies of the 12 symbionts, we concatenated five shared protein single-copy orthologs (COGs) determined by Orthofinder (Emms and Kelly, 2019) to build a "coevolutionary matrix" (list of used COGs provided in **Supplementary Table 3D**). The sequences were aligned in MAFFT v.7.450 with E-INS-i settings and processed by Gblocks using the less stringent options. The resulting matrix consisted of 1,150 amino acids. For the phylogenetic reconstructions we used ML and BI methods. The ML tree was reconstructed with 100 bootstrap replicates using the web-based PhyML with best fitting model determined by AIC using SMS function of the online PhyML server v.3.0 (HIVb + G + F). The BI trees were inferred using MrBayes v.3.2.7 under JTT + G + F evolutionary model selected by jModelTest2 v.2.1.10 (Darriba et al., 2012). Four chains were run for 10,000,000 generations and chain convergences were checked in Tracer v.1.6 and evaluated by standard deviation split (<0.01) and PSRF+ (reached the value 1.0). Both the host and the symbiont trees were rooted to fit the *N. pacificus* topologies published in Bell et al. (2021). To evaluate the possible effect of gene number on the symbiont phylogeny (number of single-gene orthologs shared by all samples shown in **Supplementary Table 3C**), we also analyzed by ML, a taxonomically reduced matrix (11 symbiont samples), containing fifty shared protein single-copy orthologs (**Supplementary Table 3D**) determined by OrthoFinder. The sequences were aligned in MAFFT v.7.450 with E-INS-i settings and processed by Gblocks using the less stringent options. The resulting alignment included 6,379 amino acids. The ML tree was reconstructed with 100 bootstrap replicates using the web-based PhyML with the best fitting model determined by AIC using SMS function in the online PhyML server v.3.0 (Q.bird + G + F).

Phylogenetic position of the Neisseriaceae-related symbiont

To determine the phylogenetic position of the bacterium binned using CONCOCT and classified as a member of Neisseriaceae, we prepared a dataset of Neisseriales

proteomes available from the NCBI database, including the putatively closest relatives indicated by BLASTp against NCBI database (Neisseriaceae-related symbionts of lice). We used representatives from other bacterial orders as outgroups, two betaproteobacteria from the order Burkholderiales, *Acidovorax* sp. KKS102 and *Burkholderia cepacia*, one alphaproteobacterium *Rhizobium leguminosarum*, and one gammaproteobacterium, *Legionella pneumophila* subsp. *pneumophila* str. Philadelphia 1. Accession numbers of the proteomes are provided in **Supplementary Table 3E**. For the Neisseriaceae-related symbionts, we included sequences from the two most complete assemblies (N206 and N204). A concatenated amino acid matrix ("Neisseriales matrix") was constructed for 30 single-gene orthologs (COGs) identified by OrthoFinder v.2.4.0 and processed in the same manner as described above for Enterobacteriaceae symbiont matrices (list of COGs provided in **Supplementary Table 3F**). The resulting matrix consisted of 4,465 amino acids. Phylogenetic reconstructions were performed by ML and BI methods. We used PhyML for ML reconstruction using 100 bootstrap replicates and the LG + G + I + F evolutionary model as determined by AIC using SMS algorithm in the online PhyML server v.3.0. The selected LG + G + I + F evolutionary model was also used for BI tree reconstruction in MrBayes v.3.2.7. The BI analysis was run in four chains for 10,000,000 generations and the convergences of the chains were checked in Tracer v.1.6 and evaluated by standard deviation split (<0.01) and PSRF+ (reached the value 1.0).

Genomes and metabolic pathways comparison

To compare the genome structure and reveal the possible rearrangements, we performed two synteny analyses. The first comparison included concatenated contigs representing five genome drafts of the new Enterobacteriaceae symbiont. The samples were selected to maximize two parameters, coverage of the symbiont's phylogenetic diversity and high completeness of the genome draft. Since several contigs contained only a single rRNA gene, and their position could not be reliably assessed, they were not included in the set. The second analysis compared these five genomes with the closest relative, i.e., *Puchtella* sp. str. PRUG (NZ_LKAS01000001.1 + NZ_LKAS01000002.1). The analyses were carried out using two programs, Mauve (Darling et al., 2010) and Clinker (Gilchrist and Chooi, 2020). Average nucleotide identity (ANI) of the genomes included in synteny analyses was calculated using a web-based ANI calculator (Yoon et al., 2017). Accession numbers for the corresponding genomes are provided in **Supplementary Table 3G**.

Assessment of metabolic capacities was based on the most complete and least fragmented genome drafts of the Enterobacteriaceae (L207) and Neisseriaceae (N206) symbionts. The Enzyme Commission (EC) numbers from the PROKKA output and the KEGG orthologs (KO) assigned by BlastKOALA (Kanehisa et al., 2016) were used to map the metabolic pathways in KEGG Mapper (last accessed: November 2021).[1] For the pathways assumed to potentially play a role in the symbiosis, absence of the genes identified as missing was verified using BLAST search. The metabolic reconstructions of B vitamins for the symbionts were compared to the metabolic pathways of several other louse symbionts with available genomes in NCBI, i.e., *Puchtella* sp. str. PRUG (NZ_LKAS01000001.1 + NZ_LKAS01000002.1), *Riesia pediculicola* USDA (GCF_000093065.1), *Legionella polyplacis* (GCA_002776555.1), *Sodalis*-like endosymbiont of *Proechinophthirus fluctus* (GCA_001602625.1), Neisseriaceae bacterium PsAf (GCA_017114885.1), and Neisseriaceae bacterium HaMa (GCA_016864895.1). The KO numbers for compared genomes are deposited in Mendeley Data under the doi link https://doi.org/10.17632/cks86467mv.6.

Proposal for the genus and species name of Enterobacteriaceae symbiont

As the results of our phylogenetic analyses show that Enterobacteriaceae symbionts form a new monophyletic lineage within the Enterobacteriaceae family, we propose the name for all included strains as "*Candidatus* Lightella neohaematopini," gen. nov., sp. nov. (hereafter, *Lightella neohaematopini* for a simple reference). The genus name *Lightella* refers to evolutionary biologist Jessica E. Light, who devoted part of her scientific career to phthirapteran research, and the species name *neohaematopini* refers to the genus of its insect host, *Neohaematopinus pacificus*.

Results and discussion

Taxonomic profiling of metagenomic reads and metagenomic bins

Initial rapid taxonomic assignment of metagenomic reads provided the first insight into the bacterial content of 21 SRA datasets. On average, only 0.4% reads from each dataset were assigned to Bacteria detecting altogether 359 unique bacterial strains (**Supplementary Table 1**). At the genus level, the highest proportion of bacterial reads were consistently classified to *Cutibacterium*, *Lactobacillus*, *Staphylococcus, and Bradyrhizobium*. In some of the samples, i.e., SRR12483201, SRR12483218, and SRR12483221, a number of bacterial reads were assigned to known symbiotic taxa, i.e., *Buchnera*,

1 http://www.genome.jp/kegg/mapper.html

Baumannia, and *Evansia* (**Supplementary Figure 2**). While the omnipresent taxa (*Cutibacterium*, *Lactobacillus*, *Staphylococcus*, *and Bradyrhizobium*) likely represent common human, environment or extraction kit related contamination typical for low biomass samples (Salter et al., 2014), identification of reads classified as obligate insect symbionts point out the association with similarly AT-rich bacteria.

The metagenomic assemblies reflected different qualities, sequencing depth and length of 21 SRA datasets (**Table 1**). The binning process resulted in a wide range in the number of bins (18–166) generated from each of the assemblies. Only 21 bins from 14 datasets were taxonomically assigned to Bacteria. The low number of bacterial bins (among 1,920 retrieved), further referred as MAGs, can be explained by the default settings used for the binning process (including min. contig length of 1,000 nt) and/or by the metagenomic origin of the SRA datasets containing high proportions of eukaryotic contigs. In contrast to the metagenomic taxonomic profiling of raw reads suggesting the omnipresence of *Cutibacterium*, *Lactobacillus*, *Staphylococcus*, *and Bradyrhizobium* genera, we have only retrieved MAGs for *Bradyrhizobium* and *Lactobacillus* from four of the meta-assemblies. It is difficult to determine the causes of this discrepancy, but it could be due to contamination with fragmented DNA, biased amplification during library preparation, or simply assignment inaccuracies stemming from the classifiers and databases we used. The majority of MAGs (12) assembled from twelve samples were identified as Enterobacteriaceae. While for seven of those the taxonomy was only resolved to the family level, five bins were assigned to symbiotic genera, i.e., *Puchtella* and *Wigglessworthia*. In addition, a single MAG representing another potentially symbiotic associate was identified as the family Neisseriaceae in a single dataset. With respect to the focus of this study, the two bacterial groups (Enterobacteriaceae and Neisseriaceae) were of particular interest and were included in further analyses.

Lightella neohaematopini: A new lineage of Enterobacteriaceae symbiont

Using BLASTx and BLASTn searches, the sequences of the new enterobacterial lineage were detected in all except one sample (SRR12483202), but the quality of these genome drafts (i.e., number of contigs and their overall lengths) varied among the SRA datasets (**Table 2**). The size comparisons indicate that the best drafts represent almost complete genomes, as with the growing quality of the assemblies, the drafts converge to the same size (**Table 2**). For eight assemblies, the size of the drafts ranged between 463,996 and 461,635 bp, with the longest contigs in the five best assemblies exceeding 217 kb. While this convergence suggested that the drafts reached a high level of completeness, BUSCO produced very low values. Depending on the database, completeness for sample L207 was estimated as 45.7, 41.3, and 45.2% for Proteobacteria, Gammaproteobacteria, and Enterobacteriales, respectively (**Supplementary Table 4**). Such low values, however, are common even for complete genomes of some symbiotic bacteria (*Candidatus* Riesia pediculischaeffi PTSU: 59.5%; *Sulcia muelleri* strain GWSS: 22.8%; values taken from the UniProt database).[2] The least fragmented assembly L207 (SRA sample SRR12483207) contained five contigs. This remaining fragmentation seems to be caused partly by the presence of two copies of the 23S rRNA gene, which were chimerically assembled in a single contig and could not be separated using this data (**Supplementary Figure 3**). This fact is also reflected by the presence of a short contig containing only the 23S rRNA gene, with coverage almost twice as high as that in the other contigs. Summarizing these parameters, the five least fragmented and most complete drafts (**Table 2**) were considered almost complete genomes and were used in the subsequent comparative analyses. The overview provided in the **Table 3** shows that this bacterium (represented by the L207 genome assembly) possesses features similar to the closest assigned taxon, *Puchtella* sp. str. PRUG (also see below for the phylogenetic analyses and **Supplementary Figure 4D** for 16S rRNA gene similarities), e.g., genome size, GC content, coding density, number of protein coding sequences, absence of transposases, mobile elements or phage-relates sequences. In contrast to this similarity and phylogenetic relatedness, virtually no synteny was detected between the *L. neohaematopini* (L207) and *Puchtella* sp. str. PRUG genomes. The dotplot produced by MUMMER v.3 (Kurtz et al., 2004), shown in **Supplementary Figure 4**, indicates that during their diversification from a common ancestor, one or both lineages underwent substantial genome rearrangements.

Annotation of the genome drafts resulted in similar sets of genes for the five best assemblies (annotations for corresponding genomes are deposited in Mendeley Data under the "doi" link https://doi.org/10.17632/cks86467mv.6). The number of protein coding genes varied between 435 and 443 (415–423 for genes with functional annotations and 19–23 for hypothetical genes). In three strains, the annotation contained two copies of 16S rRNA gene. In two strains the second copy was missing, most likely because of its position at the end of a contig (**Supplementary Figure 4**). Only one copy of 23S rRNA gene was found in the metagenomic assemblies and was placed on a short separated contig. Identical fragments of its sequences were, however, detected at the ends of two different contigs, suggesting that the 23S rRNA gene is also present in two copies and is one of the causes of the genome fragmentation (**Supplementary Figure 3**). The alignments obtained by Clinker and Mauve implemented in Geneious revealed the completely syntenic

2 www.uniprot.org/proteomes

10.3389/fmicb.2022.900312

TABLE 2 Lengths of symbiotic contigs extracted from *Neohaematopinus* lice meta-assemblies.

Neohaematopinus pacificus sample name	SSR data code	*Lightella neohaematopini draft genome code*	Number of *Lightella neohaematopini* contigs	Total length of *Lightella neohaematopini* contigs (bp)	Length of the longest *Lightella neohaematopini* contig	Neisseriaceae* symbiont draft genome Code	Number of Neisseriaceae* symbiont Contigs	Total length of Neisseriaceae* symbiont contigs (bp)	Length of the longest Neisseriaceae* Symbiont contig
DZTM1119Np	SRR12483222	L222	14	463,996	105,366	N222	3	6,579	3,880
DZTM377Np	SRR5088469	L469	7	463,572	217,438	N469	14	8,463	2,334
DZTM1701Np	SRR12483220	L220	7	463,522	188,166	N220	7	1,722	283
MVZ225305Np	SRR12483207	L207	5	462,938	217,084	N207	1	276	276
NK217036N	SRR12483201	L201	6	462,716	216,235	N201	24	13,211	5,588
DZTM1620Np	SRR12483221	L221	6	462,700	216,229	N221	7	1,765	290
DZTM2189Np	SRR12483218	L218	45	462,561	37,759	–	–	–	–
ZM.13956Np	SRR12483208	L208	6	461,635	216,026	N208	5	1,247	297
DZTM230Np	SRR12483217	L217	102	451,834	27,981	N217	5	1,314	282
ZM.13998Np	SRR12483209	L209	142	419,545	10,090	N209	6	1,484	278
DZTM2717Np	SRR12483214	L214	205	395,449	7,732	N214	6	2,034	689
MSB84515Np	SRR12483204	L204	105	347,616	11,111	N204	653	620,299	11,296
DZTM268Np	SRR12483215	L215	223	102,538	2,639	N215	30	7,499	295
DZTM708Np	SRR12483211	L211	231	92,062	1,150	N211	1	249	249
NK181766Np	SRR12483203	L203	144	51,326	1,557	–	–	–	–
DZTM203Np	SRR12483219	L219	134	39,424	660	N219	7	1,835	291
DZTM946Np	SRR12483210	L210	75	21,336	734	–	–	–	–
DZTM2776Np	SRR12483213	L213	3	12,058	1,097	N213	31	8,034	346
MVZ225310Np	SRR12483206	L206	46	11,224	3,732	N206	459	1,476,030	17,945
DZTM584Np	SRR12483212	L212	7	1,740	284	N212	4	1,058	307
NK215220Np	SRR12483202	–	–	–	–	–	–	–	–

The most complete and least fragmented genome drafts of *L. neohaematopini* are highlighted in green. The *L. neohaematopini* samples are ordered according to the total length of the concatenated genome drafts. *Neisseriaceae-related.

nature of the five strains (**Supplementary Figure 4**). Although the fragmentation into the contigs (up to seven) does not allow for an alignment in a strict sense, the complete syntenies along all contigs suggests that there has not been any rearrangement of the gene order. The few mismatches in the alignments were caused by the following: (1) Aligning inaccuracy at the ends of the contigs, (2) missing gene predictions or different annotations, and (3) real differences in absence/presence of the gene. In some cases, these causes are not easily recognized (e.g., missing annotation *vs.* absent sequence), but they account for only a small fraction of the genomic content (406 genes are present and identically annotated in all five genomes).

The BLAST-based verification of the common origin of all selected contigs produced hits of a broad taxonomic range. Although altogether the analysis supported *L. neohaematopini* as a member of Enterobacteriaceae, due to the aberrant nature

10.3389/fmicb.2022.900312

TABLE 3 Comparison of the main characteristics of the two new symbionts (the best drafts) with their closest relatives.

Genome	Louse host	Mammal host	Genome size (bp)	GC content (%)	Coding density (%)	CDS	Predicted proteins	Hypothetical proteins	Pseudogens	Transposases	Phage-related sequences	Mobile elements	BUSCO evaluation (%)
Lightella neohaematopini (L207)	*Neohaematopinus pacificus*	*Tamias alpinus*	462,938	22.2	90.5	449	443	23	29	0	0	0	45.2
Puchtella sp. str. PRUG	*Pedicinus badii*	*Procolobus rufomitratus*	558,122	24.2	92.4	602	547	34	47	0	0	0	72.7
Neisseriaceae-related symbiont (N206)	*Neohaematopinus pacificus*	*Tamias obscurus*	1,476,030	33.7	75.7	1,501	1,472	515	133	2	2	0	62.2
Neisseriaceae-related symbiont (PsAf)	*Polyplax serrata*	*Apodemus flavicollis*	1,814,374	33.7	89.4	1,739	1,660	336	106	5	0	0	85.8
Neisseriaceae-related symbiont (HaMa)	*Hoplopleura acanthopus*	*Microtus arvalis*	1,607,498	33.4	83.5	1,369	1,303	207	11	11	0	0	80.9

of the sequences (manifested by very low GC content of 22.2% and long branches in phylogenetic trees), it failed to reveal a specific closely related bacterium. Instead, the best hits included various taxa of Enterobacteriaceae, often the highly modified symbiotic bacteria such as *Buchnera*, *Blochmannia*, *Baumannia*, *Wigglessworthia*, and *Sodalis* (BLASTx results for L207 presented in **Supplementary Table 2**).

Phylogenetic position of *Lightella neohaematopini*

Considering different qualities of the genome drafts, we performed phylogenetic analyses with two different data sets. The first data set of 14 genes (7,438 amino acids), which included two lineages of *L. neohaematopini* (L207, L221) and 100 (the "phylogenetic matrix") or 63 (the "reduced phylogenetic matrix") additional members of Enterobacteriales, produced a tree in which the two strains of *L. neohaematopini* formed a monophyletic cluster on a long branch, placed as a sister group to *Puchtella* (**Figure 1** and **Supplementary Figures 5–7**). This relationship was obtained by both methods, maximum likelihood and Bayesian inference (MrBayes and PhyloBayes). However, the position of these two sister taxa (*L. neohaematopini* and *Puchtella*) as well as the arrangement of other symbiont taxa differed between the analyses. While maximum likelihood and the MrBayes analyses placed the *L. neohaematopini* + *Puchtella* lineage within a large cluster of symbionts on long branches (**Supplementary Figures 6**, **7**), the PhyloBayes analysis split this cluster into several distinct lineages (**Figure 1** and **Supplementary Figure 5**). In this tree, *L. neohaematopini* + *Puchtella* clustered in vicinity of *Wigglessworthia* and *Blochmannia*, two obligate insect symbionts with strongly modified genomes (i.e., highly reduced and AT-rich). Since highly aberrant sequences of symbiotic bacteria are known to be affected by long-branch attraction (Charles et al., 2001; Husnik et al., 2011), the cluster of long-branched symbionts produced by the maximum likelihood and MrBayes analyses is likely to be artificial and does not reflect real phylogenetic relationships. We therefore consider the PhyloBayes-derived topology, which is also compatible with the results obtained by Husnik et al. (2011), as a more reliable reconstruction of evolutionary history. However, with the current data on louse symbionts, the phylogenetic position of long-branched *L. neohaematopini* does not allow for interpretation of its symbiotic origin. Considering the phylogenetic distance between their hosts (Light et al., 2010), it is unlikely that *L. neohaematopini* and *Puchtella* share a common lice-associated symbiotic ancestor. We rather suggest that these bacteria established their relationship independently with different louse lineages and evolved into mutualists. This view is compatible with the broader phylogenetic picture showing that sucking lice

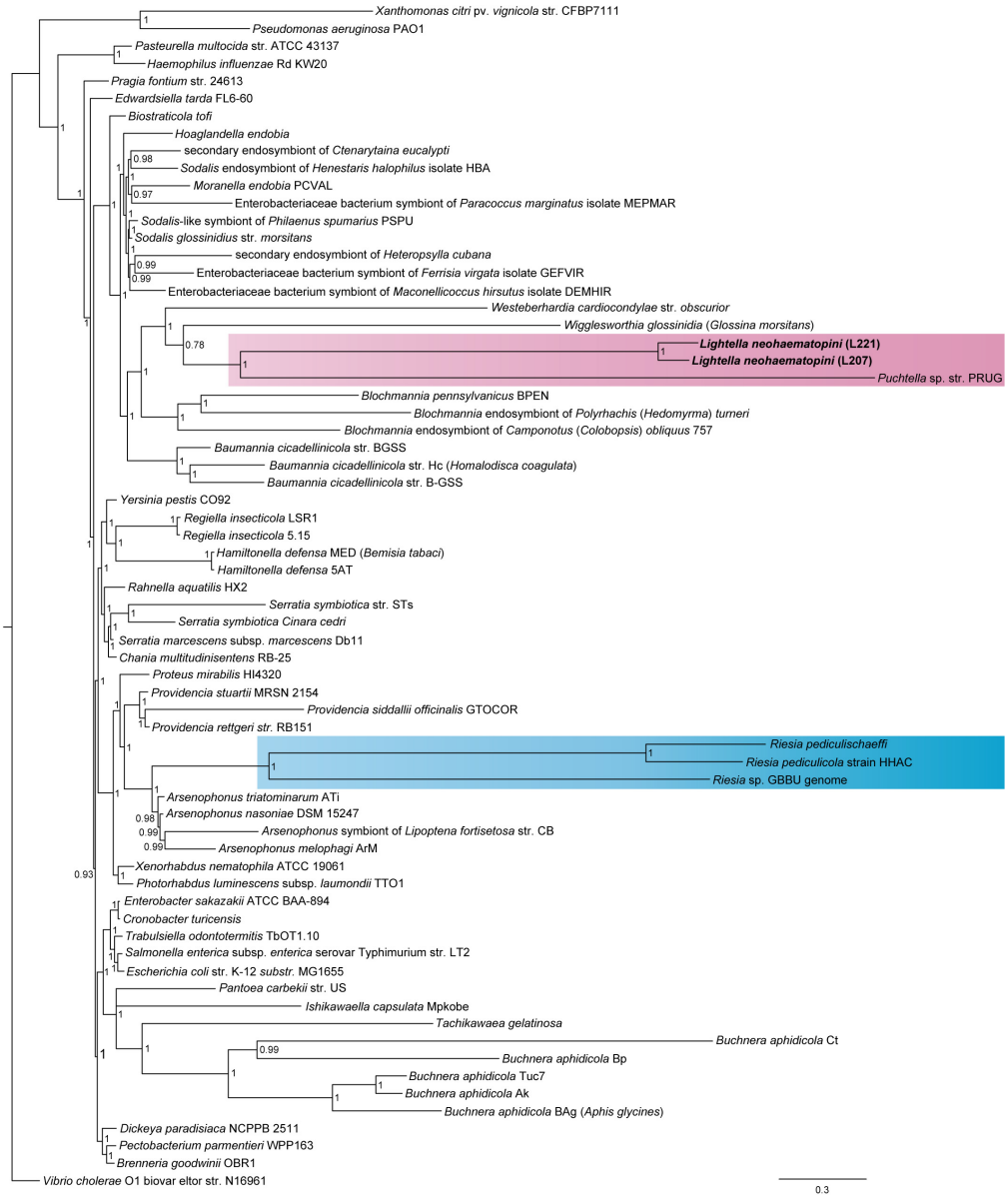

FIGURE 1
Position of *Lightella neohaematopini* within Enterobacteriales revealed by PhyloBayes analysis (BI under CAT-GTR model) of the concatenated 14-protein "reduced phylogenetic matrix" (7,438 aa). Clustering of *L. neohaemotopini* together with the *Puchtella* sp. str. PRUG is indicated by pink background. Clustering of another louse symbiont, the genus *Riesia*, within *Arsenophonus* cluster is highlighted by blue background. Values at the nodes show posterior probabilities.

acquired their symbionts several times from different groups of bacteria (Rihova et al., 2021). It should also be noted that no data are currently available on other *Neohaematopinus* species or related genera, such as *Haemodipsus* or *Sathrax*. If these lice possess symbionts related to *L. neohaematopini*, their inclusion in the analysis could in principle "break" the long branch of *L. neohaematopini* and allow for a more reliable phylogenetic placement, as well as evolutionary interpretation of its origin.

The second data set composed of 1,150 amino acids (five genes) from 12 strains of *L. neohaematopini* produced a ML tree (**Supplementary Figure 8**) and BI tree (**Figure 2**) with topologies closely corresponding to that of the host lice. Almost perfect congruence was found between the *L. neohaematopini* tree and the host tree produced by the ASTRAL-III method (**Figure 2**). The only discrepancy was the monophyly (louse tree) *vs.* paraphyly (symbionts) of the four-taxa cluster highlighted in **Figure 2**. This difference is most likely caused by an incorrect

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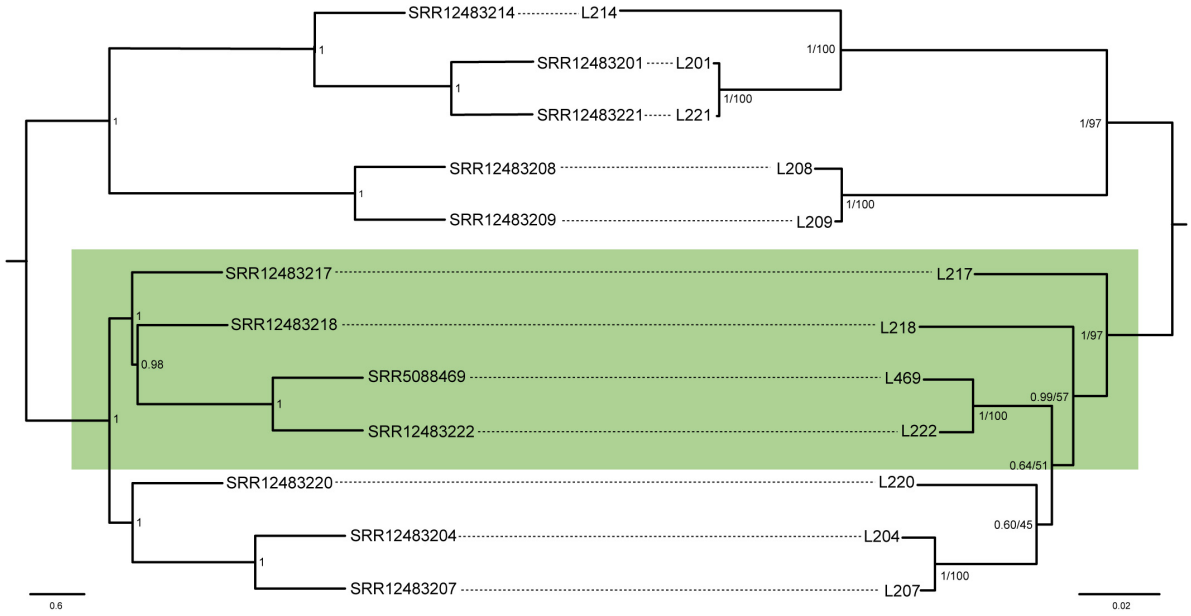

FIGURE 2
Coevolutionary reconstruction comparing phylogenetic trees of the lice and *Lightella neohaematopini*. The host tree was inferred by ASTRAL-III method from 1,107 individual gene trees, each tree derived from one of the 1,107 nucleotide matrices by IQ-TREE using GTR + F + R10 model. The symbiont tree was inferred by MrBayes (BI under JTT + G + F model) and PhyML (ML under HIVb + G + F model) from the amino-acid five-gene "coevolutionary matrix" (1,150 aa). Lengths of the branches in the symbiont tree correspond to the BI results. Values at the nodes of the symbiont tree show posterior probabilities/bootstrap. Values at the nodes of the host tree show local posterior probabilities. The green box highlights the conflicting parts of the phylogenies.

topology of one or both trees. As indicated in the symbiont tree in **Figure 2**, the conflicting phylogeny included two branches (indicated by green background in **Figure 2**) which are among the shortest in the tree and supported with low bootstrap values and posterior probabilities. Similarly, the host trees produced by two different methods (ASTRAL-III and IQ-TREE) differ in arrangement of these groups (**Figure 2** and **Supplementary Figure 9A**). The extension of the ortholog number by reducing the set of symbiont samples to 11 (L204 removed) resulted in slightly different topology but did not produce complete louse-symbiont congruence (**Supplementary Figure 9B**). Despite these minor incongruences, most likely caused by phylogenetic artifacts, the overall close correspondence between the host and the symbiont tree provides strong evidence for the common origin of these symbionts and their coevolution with the host.

Metabolic capacity and symbiotic role of *Lightella neohaematopini*

The genome of *L. neohaematopini* shows several features typical for obligate mutualists in insects (e.g., low GC content, reduced size), indicating a possible metabolic role in its louse host. In correspondence with the strong genome reduction, this new symbiont has considerably limited metabolic capacity (**Figure 3** and **Supplementary Figure 10**). Two categories of metabolites are most often considered compounds provided by an obligate symbiont to the blood-sucking host, amino acids and B vitamins (Nogge, 1981; Hosokawa et al., 2010; Snyder et al., 2010; Rihova et al., 2017). The *Neohaematopinus*-symbiont seems to have lost capacity for synthesis of most of the amino acids, only retaining pathways for lysine, glycine, and serine (for the serine/glycine pair, a category of "cyclic pathway" was established in Rihova et al. (2021), as many symbionts have the capacity for interconverting between them but cannot synthesize these amino acids from glucose; **Supplementary Figure 10**). On the other hand, *L. neohaematopini* retained considerable numbers of genes involved in synthesis of several B vitamins, namely riboflavin, folate, biotin, pantothenate, and pyridoxine (**Figure 3**). Of these vitamins, *L. neohaematopini* contains complete pathways for biotin. This pathway is also retained by three other louse-associated symbionts included in the comparison, *Puchtella* sp., *Riesia pediculicola*, and *L. polyplacis*, suggesting that biotin might be the most important compound provided to lice by their symbionts. In the riboflavin pathway, one gene is missing (5-[amino-6-(5-phospho-D-ribitylamino)uracil phosphatase; EC: 3.1.3.104] but as shown previously (and also here in **Figure 3**), this gene is missing in otherwise complete pathways of many other symbiotic bacteria, and it is thus likely that its function can be replaced by some other not yet identified gene (Rihova et al., 2021). The pathways for two additional B vitamins, folate and pyridoxine,

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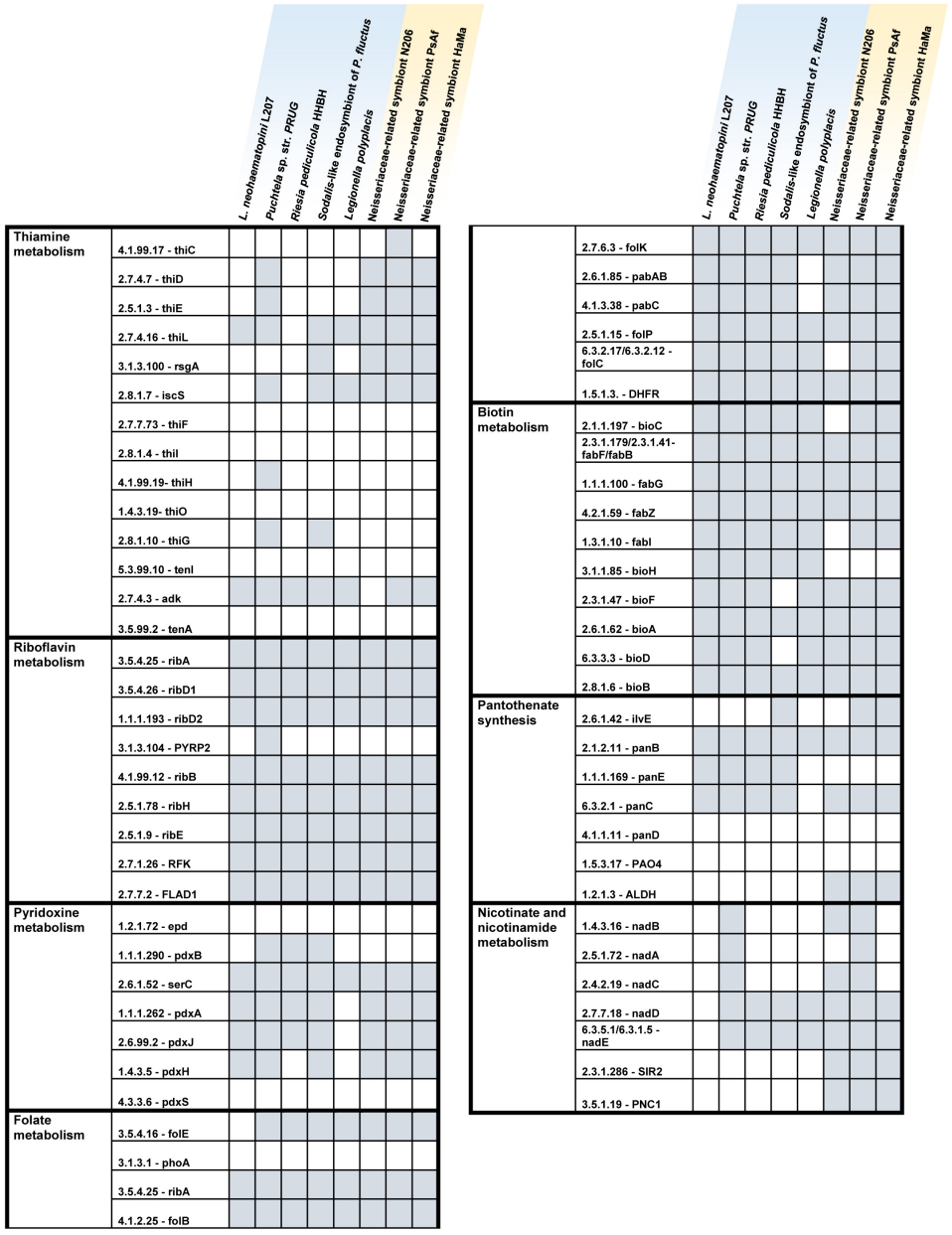

FIGURE 3
Comparison of B-vitamins pathways for *Lightella neohaematopini* L207, Neisseriaeceae-related symbiont N206 and other louse symbionts (blue background = γ-proteobacteria, yellow background = β-proteobacteria). Presence of the particular genes is indicated by gray background.

are missing two and three genes, respectively, some of them missing in all genomes included in the analysis. It is difficult to deduce from this pattern if *L. neohaematopini* can produce (or contribute to production) of these vitamins. It has been previously shown that some seemingly missing genes in these pathways playing role in mutualistic relationships can be present on a plasmid (Kirkness et al., 2010). However, this does not seem to be the case here: when using orthologs of these genes as BLAST query, we did not find their homologs in any of the 21 analyzed assemblies. For pantothenate synthesis, the symbiont's genome contains three genes (panB, panC, and panG) while the absence of the panD is likely to be compensated by the host (Price and Wilson, 2014). These pathways also do not contain any of the genes identified as potentially being pseudogenized. Altogether, this overview indicates a role for the symbiont in provisioning several B vitamins, or at least biotin, like some other louse symbionts (Boyd et al., 2017; Rihova et al., 2017, 2021). A comparison with its close relative, *Puchtella* sp. str. PRUG (symbiont of *P. baddi* from red colobus monkey *Procolobus rufomitratus*),

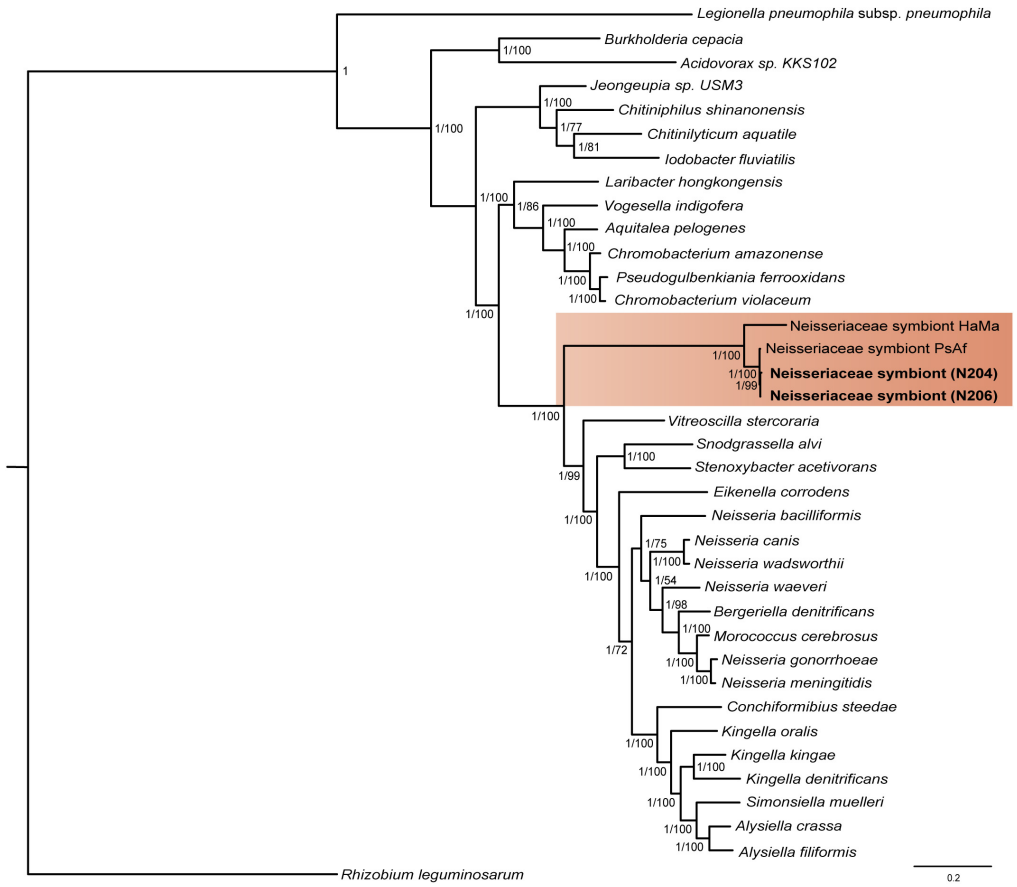

FIGURE 4
Clustering of the N206 and N204 symbionts together with the Neisseriaceae-related symbionts from Rihova et al. (2021), indicated by orange background. The tree was inferred by MrBayes (LG + G + I + F model) and PhyML (LG + G + I + F model) from the amino-acid 30-gene "Neisseriales matrix" (4,465 aa), lengths of the branches in the tree of the symbiont correspond to the BI tree results. Values at the nodes of the symbiont tree show posterior probabilities/bootstrap supports.

shows highly similar metabolic capacities of these two louse symbionts (**Figure 3**).

Neisseriaceae-related symbionts

Apart from *L. neohaematopini*, the binning process detected a putative Neisseriales-related symbiont (**Table 1**). Its sequences were present in 17 assemblies, most of them in highly fragmented and incomplete form (**Table 2**). The most complete genome draft (N206) is 1,476,030 bp long (the longest contig reaching 17,945 bp) with a low GC content (33.7%). It contains 1,472 predicted protein coding genes, 515 of them annotated as hypothetical proteins (**Table 3**). The two assemblies (N206 and N204) included in the phylogenetic analysis cluster together with the Neisseriaceae-related symbionts PsAf and HaMa previously described from *Polyplax serrata* and *Hoplopleura acanthopus* lice, respectively (Rihova et al., 2021; **Figure 4**). The HaMa was also retrieved as the first BLAST hit when the contigs assigned to Neisseriaceae in the 17 assemblies were used as a query against the nt NCBI database. The clustering on a long common branch and high branch support (both ML and BI analyses) suggest that all these Neisseriaceae-related bacteria may have originated from a single symbiotic ancestor (**Supplementary Figure 11** and **Figure 4**). This phylogenetic position and the features typical for bacteria undergoing adaptation to symbiosis (e.g., low GC content and high number of pseudogenes) indicate the symbiotic nature of these bacteria. The main characteristics of the N206 genome, and its comparison to the other Neisseriaceae-related symbionts are summarized in **Table 3**. Since *N. pacificus* harbor *L. neohaematopini* as a typical obligate mutualistic P-symbiont, the significance of the Neisseriaceae-related symbiont for the host is unclear. Furthermore, the highly fragmented and possibly incomplete genome assembly (BUSCO completeness value 62.2%) does not allow for a reliable reconstruction of its metabolic capacities (in **Figure 3** and **Supplementary Figure 10** we show the capacities which can be inferred from this data).

It is, however, interesting to see that these bacteria are present in several louse genera, possibly playing the role of either S-symbionts (*N. pacificus, P. serrata*) or even P-symbionts (*H. acanthopus*).

The results presented in this study provide two pieces of information relevant for broader insight into the evolution of symbiosis between lice and bacteria. First, the new putative obligate mutualist, *L. neohaematopini*, extends the list of bacterial lineages which have established mutualistic symbiosis with different groups of sucking lice. This further supports the view that sucking lice underwent an exceptionally dynamic process of symbiont acquisitions and replacements. Phylogenetic match of *L. neohaematopini* with *Neohaematopinus pacificus* lice from different chipmunk species provides strong evidence that these symbionts were acquired in a single evolutionary event and have been maintained during the host's radiation. This codiversification process likely reflects the nutritional role of these bacteria. A comparison with other known louse symbionts suggests that the compounds most likely responsible for this dependence are several B vitamins. In contrast, the second potentially symbiotic bacterium is related to Neisseriaceae-related symbionts already known from two different groups of lice (Rihova et al., 2021). Interestingly, in *Neohaematopinus pacificus* lice the co-occurrence of these two bacteria resembles the situation in *Polyplax serrata*, where the mutualistic *Legionella polyplacis* is occasionally accompanied by the Neisseriaceae-related symbiont. We expect that addition of so far unexplored louse lineages will further extend overall diversity of their symbionts, but it can also help to resolve some of the current phylogenetic and evolutionary uncertainties.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

VH, EN, and JŘ conceived the study. All authors contributed to the data analyses, interpretation of the results and writing the manuscript.

Funding

This work was supported by the Grant Agency of the Czech Republic (grant GA20-07674S to VH).

Acknowledgments

We would like to acknowledge Jessica E. Light and Julie Allen for their reviews of the article. We also highly appreciate access to computing and storage facilities owned by parties and projects contributing to the National Grid Infrastructure MetaCentrum provided under the programme "Projects of Large Research, Development, and Innovations Infrastructures" (CESNET LM2015042).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.900312/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 08 August 2022
DOI 10.3389/fmicb.2022.962252

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

RECEIVED 06 June 2022
ACCEPTED 08 July 2022
PUBLISHED 08 August 2022

CITATION
Nishide Y, Oguchi K, Murakami M, Moriyama M, Koga R and Fukatsu T (2022) Endosymbiotic bacteria of the boar louse *Haematopinus apri* (Insecta: Phthiraptera: Anoplura). *Front. Microbiol.* 13:962252. doi: 10.3389/fmicb.2022.962252

Endosymbiotic bacteria of the boar louse *Haematopinus apri* (Insecta: Phthiraptera: Anoplura)

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Insects exclusively feeding on vertebrate blood are usually dependent on symbiotic bacteria for provisioning of B vitamins. Among them, sucking lice are prominent in that their symbiotic bacteria as well as their symbiotic organs exhibit striking diversity. Here we investigated the bacterial diversity associated with the boar louse *Haematopinus apri* in comparison with the hog louse *Haematopinus suis*. Amplicon sequencing analysis identified the primary endosymbiont predominantly detected from all populations of *H. apri* with some minor secondary bacterial associates. Sequencing and phylogenetic analysis of bacterial 16S rRNA gene confirmed that the endosymbionts of the boar louse *H. apri*, the hog louse *H. suis* and the cattle louse *Haematopinus eurysternus* form a distinct clade in the Gammaproteobacteria. The endosymbiont clade of *Haematopinus* spp. was phylogenetically distinct from the primary endosymbionts of other louse lineages. Fluorescence *in situ* hybridization visualized the endosymbiont localization within midgut epithelium, ovarial ampulla and posterior oocyte of *H. apri*, which were substantially the same as the endosymbiont localization previously described in *H. suis* and *H. eurysternus*. Mitochondrial haplotype analysis revealed that, although the domestic pig was derived from the wild boar over the past 8,000 years of human history, the populations of *H. apri* constituted a distinct sister clade to the populations of *H. suis*. Based on these results, we discussed possible evolutionary trajectories of the boar louse, the hog louse and their endosymbionts in the context of swine domestication. We proposed '*Candidatus* Haematopinicola symbiotica' for the distinct clade of the endosymbionts of *Haematopinus* spp.

KEYWORDS

***Haematopinus apri*, boar louse, *Haematopinus suis*, hog louse, symbiont, evolution, domestication, symbiotic organ**

Introduction

Insects represent the majority of the biodiversity in the terrestrial ecosystem (Grimaldi and Engel, 2005), in which symbiotic microorganisms generally play important biological roles (Buchner, 1965; Bourtzis and Miller, 2003). In particular, insects utilizing nutritionally recalcitrant food resources are usually dependent on their specific microbial partners, wherein the representative microbial functions are digestion of plant cell wall polymers that are otherwise indigestible for many wood-feeding insects like termites, bark beetles, etc. (Brune, 2014; Biedermann and Vega, 2020), provisioning of essential amino acids that are lacking in plant vascular fluid for plant sap-sucking insects like aphids, cicadas, etc. (Moran et al., 2008; Douglas, 2009), and the synthesis of B vitamins that are deficient in vertebrate blood for blood-sucking insects like lice, bedbugs, etc. (Rio et al., 2016; Husnik, 2018).

Blood-sucking insects, such as mosquitoes, fleas, tsetse flies, lice, bedbugs, etc., are notorious not only for causing itch, wound, and inflammation to human skin but also for vectoring devastating human and veterinary pathogens (Lehane, 2005). Among them, those feeding on vertebrate blood throughout their life stages usually develop specialized symbiotic organs for hosting specific symbiotic bacteria: *Wigglesworthia* in tsetse flies (Aksoy, 1995; Akman et al., 2002), *Aschnera* in nycteribiid bat flies (Hosokawa et al., 2012), *Wolbachi*a in bedbugs (Hosokawa et al., 2010; Nikoh et al., 2014), and others.

Sucking lice (Phthiraptera: Anoplura) live on vertebrate blood as the sole food source throughout their life cycle (Lehane, 2005), and most of them develop specialized symbiotic organs that harbor specific symbiotic bacteria (Ries, 1931; Buchner, 1965). Both histological inspection (Ries, 1931) and molecular phylogenetic survey (Hypša and Křížek, 2007) revealed that their symbiotic organs and associated bacterial symbionts are strikingly diverse among different lice lineages and likely of independent evolutionary origins: '*Candidatus* Riesia spp.' are harbored in a distinct oval symbiotic organ located on the ventral side of the intestine called the "stomach disc" in human and primate lice *Pediculus* spp. and *Pthirus* spp. (Sasaki-Fukatsu et al., 2006; Allen et al., 2007; Perotti et al., 2007; Kirkness et al., 2010; Boyd et al., 2014); '*Candidatus* Puchtella pedicinophila' is harbored in a specific region of the midgut epithelium in the monkey lice *Pedicinus* spp. (Fukatsu et al., 2009; Boyd et al., 2017); and '*Candidatus* Legionella polyplacis' is in an intestine-associated symbiotic organ in a rodent louse *Polyplax serrata* (Říhová et al., 2017). In other lice lineages, the symbiotic organs are histologically not so distinct and the symbiotic associations might be either younger and/or casual: *Sodalis* and *Rickettsia* in a seal louse *Proechinophthirus fluctus* (Boyd et al., 2016); *Neisseria/Snodgrassella*-allied in rodent lice *Hoplopleura* spp. and *Polyplax* spp. (Říhová et al., 2021). Thus far, however, *in vivo* localization and developmental dynamics of the symbiotic organs and the symbiotic bacteria have been only briefly described in most cases, except for those in the human body louse *Pediculus humanus* and also those in the cattle louse *Haematopinus eurysternus* (Ries, 1931; Buchner, 1965).

The genus *Haematopinus* consists of some 20 species of ungulate lice, including the cattle louse *H. eurysternus*, the hog louse *Haematopinus suis*, the horse louse *Haematopinus asini*, and others (Durden and Musser, 1994). Historically, the endosymbiotic bacteria of *Haematopinus* spp. have been described as follows: the initial microscopic detection in *H. suis* (Sikora, 1919; Buchner, 1920; Florence, 1924); detailed histological inspection of *in vivo* localization and developmental dynamics in *H. eurysternus* (Ries, 1931); transmission electron microscopic observation in *H. suis* (Żelazowska and Biliński, 1999); and molecular phylogenetic analysis based on 16S rRNA gene sequences from *H. suis*, *H. eurysternus* and *Haematopinus apri* (Hypša and Křížek, 2007). These previous studies, mostly conducted on *H. suis* or *H. eurysternus*, have shown that the endosymbionts of *Haematopinus* spp. form a well-supported clade in the Gammaproteobacteria and exhibit peculiar localization patterns distinct from the other louse lineages: in both females and males, numerous bacteriocytes are scattered over the midgut epithelium, recognized as swellings into the midgut cavity, in which the symbiotic bacteria look like endocellular at a glance but actually reside in an extracellular space surrounded by the bacteriocyte cytoplasm; and specifically in females, the symbiotic bacteria also localize to the dorsal "depot bacteriomes," from which the symbiotic bacteria migrate to the ovarial ampullae and infect to developing oocytes (Ries, 1931; Buchner, 1965). These early histological works, based on conventional light microscopy and illustrated by hand-drawn sketches, should be re-examined by modern histological techniques and sophisticated microscopy.

The boar louse *H. apri* parasitizes the wild boar and distributes across Eurasia (Durden and Musser, 1994). Reflecting the recent origin of the pig (*Sus scrofa domestica*) *via* domestication of the wild boar (*Sus scrofa scrofa*) over the past 8,000 years (Larson et al., 2005; Larson and Fuller, 2014; Frantz et al., 2016), the boar louse *H. apri* is morphologically and phylogenetically similar to the hog louse *H. suis*, although the former exhibits paler body color, relatively smaller legs, and smaller paratergites in comparison with the latter (Ferris, 1933). As for the endosymbiont of *H. apri*, only a bacterial 16S rRNA gene sequence (1,213 bp in size; DQ076665), has been reported and analyzed for inferring the molecular phylogenetic placement (Hypša and Křížek, 2007), while no other biological information is available. The evolutionary relationship between *H. apri* and *H. suis* is somewhat reminiscent of the relationship between the human head louse (*P. humanus capitis*) and the human body louse (*P. humanus humanus*) that has been argued in relation to human clothing and civilization (Kittler et al., 2003; Veracx and Raoult, 2012; Amanzougaghene et al., 2020). In this context, detailed characterization of the

endosymbiont of *H. apri* in comparison with the endosymbiont of *H. suis* is of interest.

In an initial attempt toward this research goal, we investigated the bacterial diversity associated with multiple populations of the boar louse *H. apri* using amplicon sequencing, molecular phylogenetic and histological approaches, thereby identifying the primary endosymbiont and minor bacterial associates of *H. apri*. We discussed the relationship between the endosymbionts of *H. apri* and *H. suis* in the context of swine domestication, and proposed a candidate name for the endosymbiotic bacteria associated with the louse genus *Haematopinus*.

Materials and methods

Insect materials

Table 1 and Supplementary Table S1 list the insect samples examined in this study. These insects were preserved either in ethanol or acetone (Fukatsu, 1999), or brought to the laboratory alive and processed immediately. For histological observations, the insects were dissected in phosphate-buffered saline (PBS; 0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4) by using fine tweezers under a dissection microscope (M165FC; Leica).

DNA extraction, PCR, cloning and sequencing

The samples were washed by soaking in 1% bleach and 0.1% Triton X-100 for 1 min and then rinsed twice with water. Then, the samples were individually crushed using BioMasher II (Nippi, Tokyo, Japan) and subjected to DNA extraction using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Insect mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified by PCR using KOD FX Neo (TOYOBO, Osaka, Japan) with the primers modified-mtd6 (5′-GGA GGW TTY GGA AAT TGR TTA GTD CC-3′) and mtd11 (5′-ACT GTA AAY ATA YGR TGW GCT CA-3′) (Jiang et al., 2013) that were selected to match COI sequences of *H. suis* and *H. apri* (accession numbers HM241908 and KC814616). Bacterial 16S rRNA gene was amplified by PCR using Ex taq HS (TaKaRa, Shiga, Japan) with two sets of the primers 16SA1 (5′-AGA GTT TGA TCM TGG CTC AG-3′)—16SB2 (3′-CGA GCT GAC ARC CAT GCA-3′) and 16SA2 (5′-GTG CCA GCA GCC GCG GTA ATA C-3′)—16SB1 (5’-TAC GGY TAC CTT GTT ACG ACT T-3′) (Fukatsu and Nikoh, 1998). The PCR products were cloned using pGEM-T Easy Vector (Promega, Madison, United States), Mighty mix for DNA ligation (Takara) and *Escherichia coli* DH5 competent cells (Takara). The inserted plasmids were extracted from the transformed *E. coli* cells using QIAprep Spin miniprep kit (QIAGEN) and subjected to sequencing reactions using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Massachusetts, United States) with the primers M13FW (5′-GTA AAA CGA CGG CCA GT-3′) and M13RV (5′-CAG GAA ACA GCT ATG AC-3′) targeting the flanking regions of the vector.

Molecular phylogenetic analysis

The molecular phylogenetic analyses were conducted by the maximum likelihood methods using MEGA 5.2 (Tamura et al., 2011). The optimum model was selected by model tests to describe each phylogenetic tree. Bootstrap probability values based on 1,000 replications are shown on the nodes. To analyze the genetic diversity of host COI sequences, we depicted the network diagrams of COI haplotypes using MEGA-X (Kumar et al., 2018), DnaSP 6 (Rozas et al., 2017) and Network 10 (fluxus-engineering.com).

TABLE 1 Samples of the boar louse *H. apri* and the hog louse *H. suis* examined in this study.

Louse species	Host animal	Collection locality[1]	Collection date	Collector[2]	Sample ID[3]	Note
Boar louse	Wild boar					
Haematopinus apri	*Sus scrofa*	Toyama	Feb. 2008	TY	#01-#18	Fixed specimens
	leucomystax	Wakayama	Feb.-Mar. 2008	AT	#19-#33	Fixed specimens
		Oita	Mar. 2008	AT	#34-#41	Fixed specimens
		Hyogo	Nov. 2008, Mar. 2009	AT	#42-#50	Fixed specimens
		Osaka	Mar. 2008	SU	#51-#56	Fixed specimens
		Ehime	Dec. 2021	YM	#57-#58 + α	Fresh specimens
Hog louse	Domestic pig					
Haematopinus suis	*Sus scrofa*	Saitama	Jun. 2008	K	#59-#63	Fixed specimens
	domestica					

[1]All localities are in Japan.
[2]AT, Ai Takano; K, Kato; SU, Shigehiko Uni; TY, Takeo Yamauchi; YM, Yuki Miyaoka.
[3]Detailed sample information and associated sequence accession numbers are described in Supplementary Table S1.

Amplicon sequencing

The hypervariable V3/V4 region of bacterial 16S rRNA gene, around 0.4 kb in size, was targeted for amplicon sequencing analyses. Library construction was conducted by the 2-step tailed PCR procedure with the primers first_341_MIX (5′-ACA CTC TTT CCC TAC ACG CTC TTC CGA TCT NNN NNC CTA CGG GNG GCW GCA G-3′) and first_805r_MIX (5′-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TNN NNN GAC TAC HVG GGT ATC TAA TCC-3′), and then second_F (5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC AC-Indexseq (8mer)-ACA CTC TTT CCC TAC ACG ACG C-3′) and second_R (5′-CAA GCA GAA GAC GGC ATA CGA GAT-Indexseq (8mer)-GTG ACT GGA GTT CAG ACG TGT G-3′). The libraries were measured for concentration using Synergy H1 (Bio Tec, Vermont, United States) and QuantiFluor dsDNA System (Promega), and quantified using Fragment Analyzer (Agilent, California, United States) and dsDNA 915 Reagent Kit (Agilent). Sequencing of the libraries was performed using Miseq (Illumina, California, United States). From the raw amplicon sequences obtained (accession number DRR360832-DRR360847), only the reads containing the tags in PCR primers were selected using FASTX-Toolkit (ver.0.0.14). The output reads were subjected to denoising and removal of chimeric sequences using the DADA2 plugin in the Quantitative Insights Into Microbial Ecology (QIIME2) v.2021.11 pipeline (Bolyen et al., 2019). Bacterial taxa were assigned to the representative amplicon sequences based on GreenGenes V.13_8 (McDonald et al., 2012) using the q2-feature-classifier QIIME2 plugin (Bokulich et al., 2018).

Fluorescence *in situ* hybridization (FISH)

Whole-mount fluorescent *in situ* hybridization (FISH) targeting bacterial 16S rRNA was performed essentially as described previously (Koga et al., 2009). The dissected insect tissues were fixed in 4% paraformaldehyde in PBS for 3 h and then thoroughly washed in PBST (0.1% Tween 20 in PBS). The fixed insect tissues were washed twice in hybridization buffer (20 mM Tris–HCl [pH 8.0], 0.9 M NaCl, 0.01% SDS, 30% formamide). To specifically target 16S rRNA of the primary symbiont of *Haematopinus* spp., we designed the oligonucleotide probe Wbsym1181R (5′-ACC TTC GCA GGT TAG CTT-3′) labeled with fluorochrome Alexa Fluor 647 at the 5′ terminus. For universal detection of bacterial 16S rRNA, we used the probe EUB338 (5′-GCT GCC TCC CGT AGG AGT-3′) (Amann et al., 1990) labeled with Alexa Fluor 555 at the 5′ terminus. The samples were incubated in hybridization buffer containing 50 nM probe. After washed twice in PBST, host nuclear DNA and filamentous actin were stained with 4.5 μM 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) and 1.5 μM Alexa Fluor 488-labeled phalloidin (Thermo Fisher Scientific), respectively, for 1 h at room temperature. Then, the samples were washed with PBST again, mounted in 50% glycerol in PBS, and observed under a laser scanning confocal microscope (LSM 700; Carl Zeiss, Germany). As a control experiment for FISH detection, RNase digestion controls in which the tissue samples were treated with RNase A prior to hybridization were conducted (Supplementary Figure S1).

Results

Microbiome of *Haematopinus apri* and *Haematopinus suis*

In order to grasp the bacterial diversity associated with *H. apri*, 13 insects representing six collection localities (see Supplementary Table S1) were subjected to amplicon sequencing analysis targeting the hypervariable V3/V4 region of bacterial 16S rRNA gene. In addition, three insects of *H. suis* collected from a locality were analyzed in the same way (see Supplementary Table S1). Figure 1 shows the composition of the amplicon sequences for each of the insect samples. The same bacterial taxon was predominantly detected from almost all the samples (except for Hyogo samples of *H. apri*), accounting for over 85% of the detected sequences for each of the samples. The remaining minor bacterial taxa were assigned as allied to *Neisseria*, *Streptococcus*, *Serratia* and *Aeromonas* for *H. apri*, and *Turicibacter* for *H. suis* (Figure 1). Exceptionally, two Hyogo samples of *H. apri* exhibited different patterns: in a sample, *Enterobacter* was highly predominant, accounting for over 80% of the detected sequences; in another sample, not only *Enterobacter* but also *Citrobacter* occupied substantial fractions of the microbiota (Figure 1). Notably, these Hyogo samples were shipped to our laboratory by refrigerated, not frozen, courier service, delayed in arrival, and then transferred to an ethanol vial for preservation. Hence, it seems likely that proliferation of the microbes in the dead insect bodies resulted in the exceptional microbial compositions in these samples. Taken together, these results strongly suggested that a single bacterial species constitutes the major microbial associate of *H. apri* and *H. suis*, which is likely the primary endosymbiont as described in previous histological and phylogenetic studies on *H. suis* (Florence, 1924; Żelazowska and Biliński, 1999; Hypša and Křížek, 2007).

16S rRNA gene sequence diversity of primary endosymbionts among populations of *Haematopinus apri* and *Haematopinus suis*

Ten insects of *H. apri* representing four collection localities in Japan (see Supplementary Table S1) were individually subjected to PCR, cloning and sequencing of bacterial 16S rRNA gene. All the sequences were completely identical to each other (accession number LC706254). DNA database searches using the sequence as

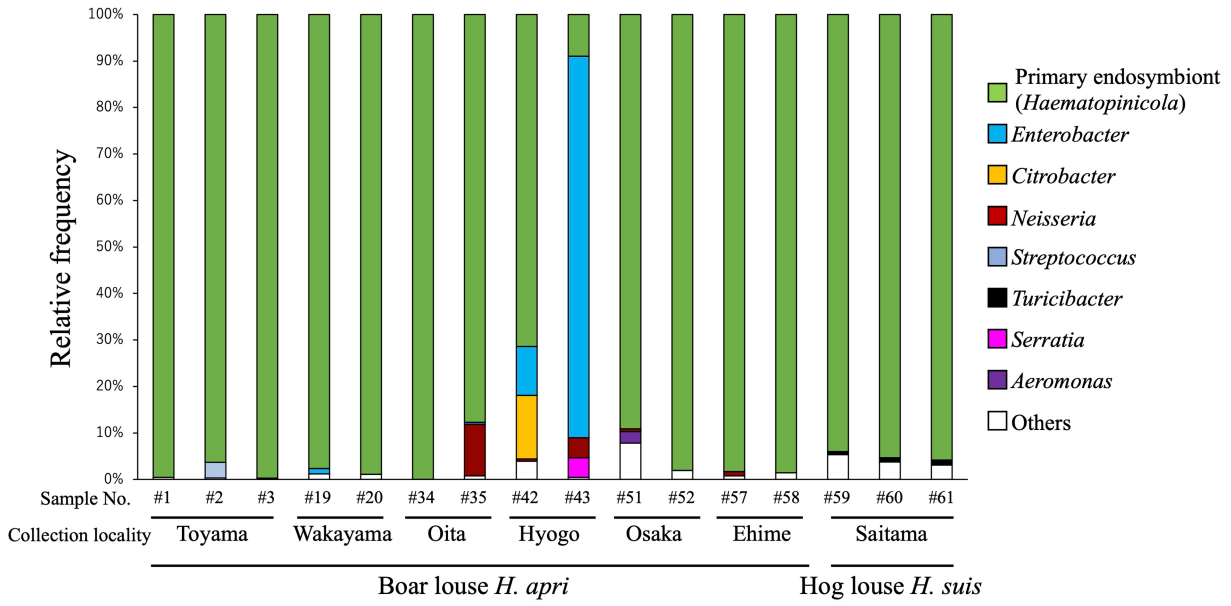

FIGURE 1
Bacterial diversity associated with the boar louse *Haematopinus apri* and the hog louse *Haematopinus suis* based on amplicon sequencing data of the hypervariable V3/V4 region of 16S rRNA gene. Assigned bacterial taxa are color-coded as shown on the right side. As for sample information and amplicon sequencing data, see Supplementary Table S1.

query retrieved 16S rRNA gene sequences of endosymbionts of *H. suis* from United States (KX146200), *H. suis* from Czech (DQ076662), *H. apri* from Czech (DQ076665) and *H. eurysternus* from Czech (DQ076661) (Hypša and Křížek, 2007; Allen et al., 2016) as top hits. In addition, two insects of *H. suis* collected from a locality in Japan (see Supplementary Table S1) were subjected to the analysis, which yielded identical sequences (accession number LC706255) with 1 bp difference from the sequence obtained from *H. apri*. Supplementary Figure S2A summarizes the sequence differences among the endosymbiont sequences of *H. apri* and *H. suis* from Japan, United States and Czech.

Molecular phylogenetic analysis of primary endosymbionts of *Haematopinus apri* and *Haematopinus suis*

Figure 2 shows the phylogenetic relationship of the primary endosymbionts of *H. apri* and *H. suis* based on 16S rRNA gene sequences. The endosymbionts of *H. apri* from Japan, *H. suis* from Japan, *H. suis* from United States and *H. suis* from Czech formed a highly supported compact clade. Reflecting some nucleotide differences (see Supplementary Figure S2A), the endosymbiont of *H. apri* from Czech was placed outside the clade, and formed a highly supported clade together. Then, the endosymbiont of *H. eurysternus* was placed outside the clade of the endosymbionts of *H. suis* and *H. apri*, forming a highly supported clade together. The distinct clade of the endosymbionts of *Haematopinus* spp. was not allied to the endosymbionts of other insects including sucking lice, such as *Riesia*, *Puchtella*, *Wigglesworthia*, *Blochmannia* and *Baumannia*, in the Gammaproteobacteria.

In vivo localization of primary endosymbiont of *Haematopinus apri*

Figure 3 shows FISH visualization of the primary endosymbiont in *H. apri*. In adult females with mature ovaries, dense symbiont signals were detected in a specialized ovarial region lying between lateral oviduct and ovarioles, so-called ovarial ampulla (Buchner, 1965) (Figures 3B,C). In both females and males, dense symbiont signals were detected throughout the midgut epithelium in a scattered manner (Figures 3D,E). Each of the patchy bacteriocyte-like structures, which contained a dense population of the symbiont cells and protruded from the epithelial wall to the midgut cavity, were actually constituted by multiple epithelial cells that encased the symbiont cells within the extracellular cavity formed at the center (Figures 3F–H). Within the ovarial ampulla, by contrast, the symbiont cells were found within the host cytoplasm endocellularly (Figures 3I,J). In mature oocytes in the ovarioles, the symbiont cells localized to the posterior pole, where infecting bacterial cells through the follicle cell layer were often observed (Figure 3K). These *in vivo* localization and infection dynamics of the primary endosymbiont were generally in agreement with previous histological descriptions on *H. eurysternus* and *H. suis* (Ries, 1931; Buchner, 1965; Żelazowska and Biliński, 1999).

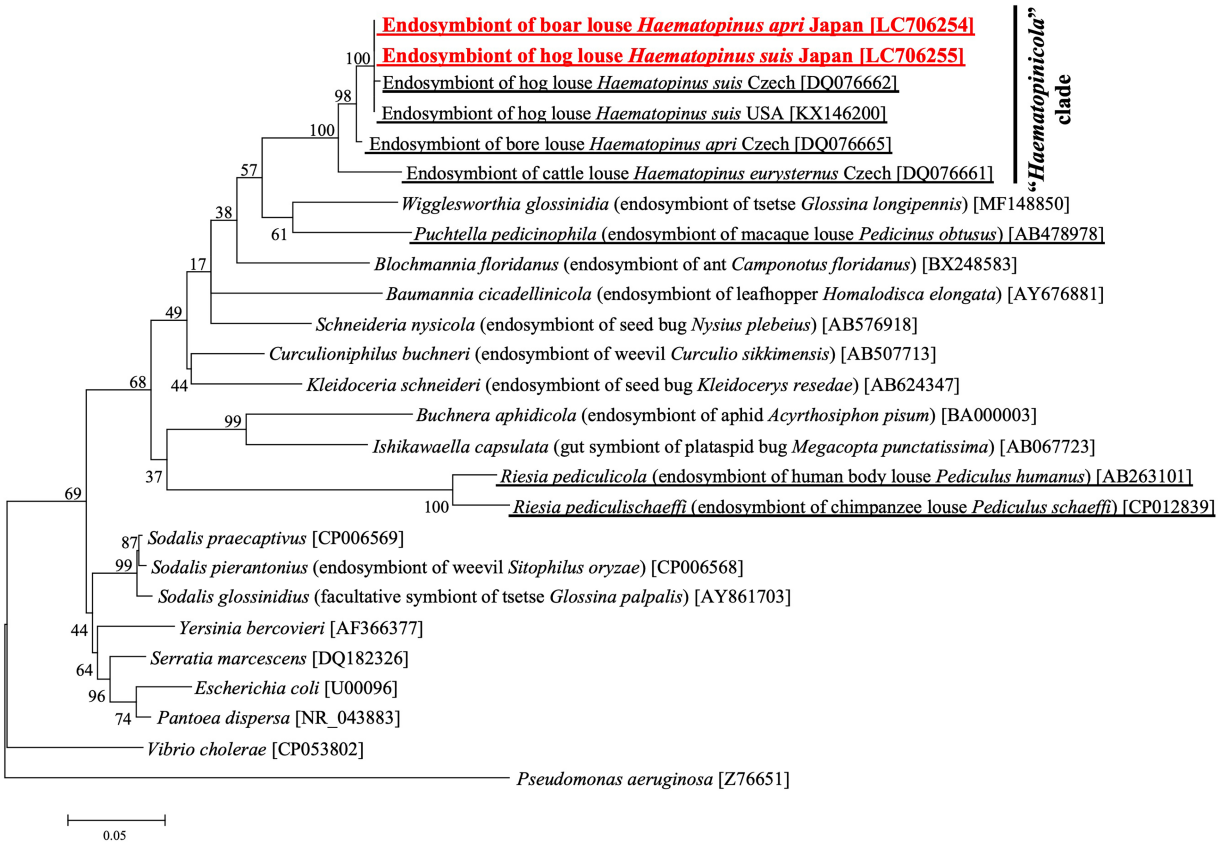

FIGURE 2
Phylogenetic placement of the primary endosymbionts of the boar louse *H. apri* and the hog louse *H. suis* based on 16S rRNA gene sequences. A maximum-likelihood phylogeny inferred from 1,013 aligned nucleotide sites is shown with bootstrap probability at each node (model: K2+G+I). Host insect information is shown in parentheses, whereas accession number is indicated in brackets. The louse endosymbiont sequences determined in this study are highlighted in red, whereas the louse endosymbiont sequences reported in previous studies are underlined.

Neisseria/Snodgrassella-allied bacteria detected from *Haematopinus apri*

Recently, microbiome survey of *Polyplax* and *Hoplopleura* rodent lice identified *Neisseria/Snodgrassella*-allied bacteria as either obligatory or facultative microbial associates (Říhová et al., 2021). Since our amplicon sequencing analysis also detected *Neisseria*-allied bacterial associates from several *H. apri* samples at low frequencies (Figure 1), we cloned and sequenced 16S rRNA gene sequence of the bacteria from *H. apri*. DNA database searches using the sequence as query retrieved 16S rRNA gene sequences of Neisseriaceae bacteria from a rodent louse *Hoplopleura acanthopus* (CP046107) and a prairie dog flea *Oropsylla hirsute* (EU137419) as the top hits, and *Snodgrassella alvi* from bumble bees and honeybees (HM108703, HM113170 and others) as the next best hits. Figure 4 shows the phylogenetic relationship of *Snodgrassella, Neisseria* and allied 16S rRNA gene sequences, which indicated that the bacterium associated with *H. apri* is certainly placed in the Neisseriaceae, and the most closely related to the bacterial associates of the rodent louse *Hoplopleura* and the prairie dog flea *Oropsylla*.

Population genetics of *Haematopinus apri* and *Haematopinus suis*

In order to understand the population genetic structure of the host lice, mitochondrial COI gene was amplified by PCR and sequenced for 44 individuals of *H. apri* originating from five collection localities and also for two individuals of *H. suis* (see Supplementary Table S1). In total, 13 haplotypes were identified for the COI gene sequences: haplotypes 1–12 represented *H. apri* and closely related to each other, whereas haplotype 13 represented *H. suis* and was distant from the other haplotypes (Figure 5A). Supplementary Figure S2B summarizes the sequence differences among mitochondrial COI gene sequences of *H. apri* and *H. suis* from Japan, China and Australia. In the DNA databases, a COI gene sequence of *H. apri* from Hyogo, Japan (KC814616), a COI gene sequence of *H. apri* from Hunan, China (ON000919), and two COI gene sequences of *H. suis* from Perth, Australia (HM241908, KC814607), were deposited. We performed molecular phylogenetic analysis of these COI gene sequences, and demonstrated that *H. apri* from Japanese and Chinese populations and *H. suis* from Japanese and Australian

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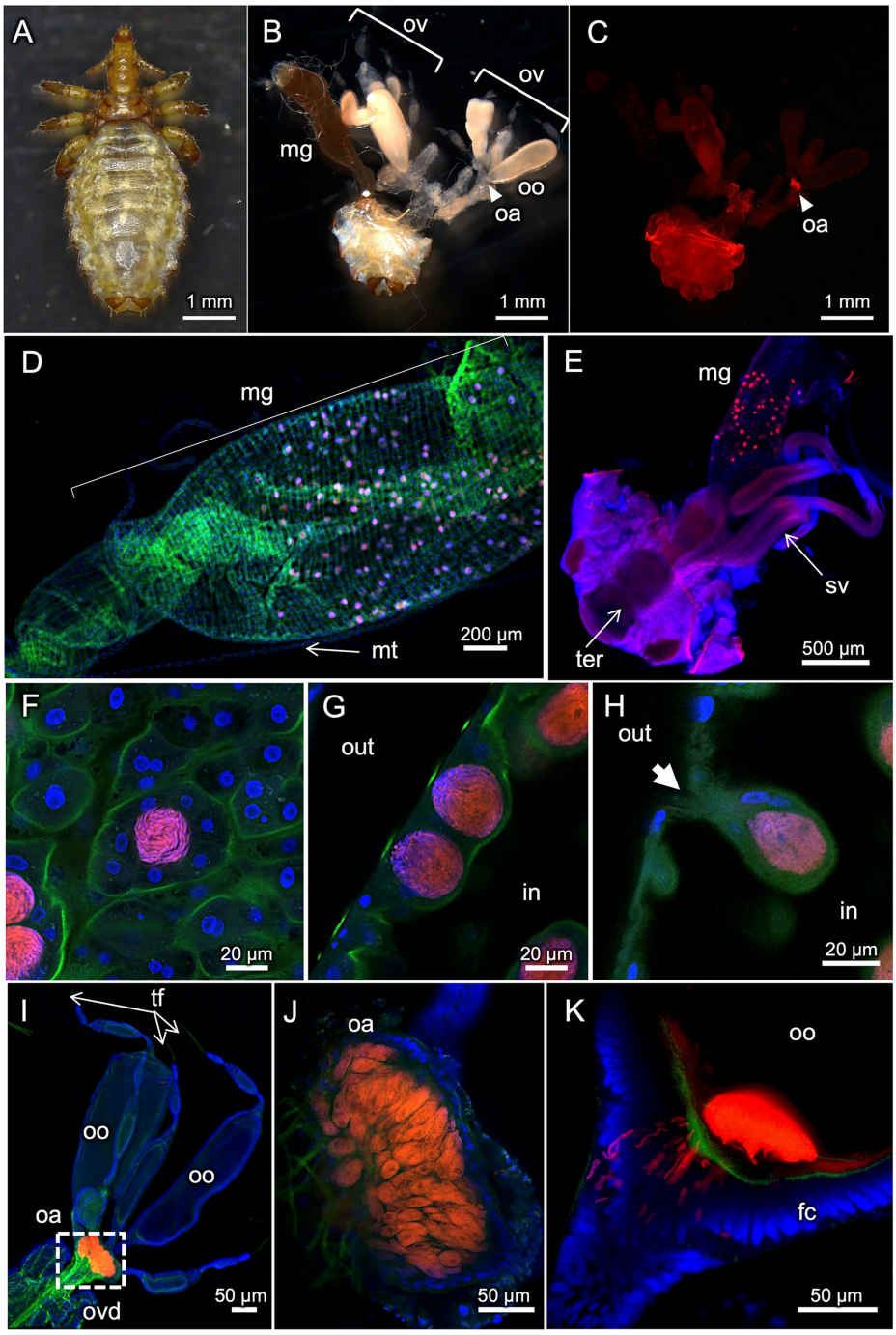

FIGURE 3
FISH visualization of *in vivo* localization of the primary endosymbiont in *H. apri*. **(A)** Dorsal view of an adult female. **(B)** Internal organs dissected from an adult female. **(C–E)** Whole-mount FISH visualization of the symbiont in dissected organs. **(C)** Localization of the symbiont in the ovarial ampulla of an adult female. The sample is the same as (**B**). **(D)** Scattered localization of the symbiont across the midgut epithelial region of an adult female. **(E)** Similar localization pattern of the symbiont in the midgut epithelium of an adult male. **(F–H)** Magnified confocal images of the symbiont in the midgut epithelium. **(F)** Optical section image parallel to the epithelial plane. **(G)** Optical section image perpendicular to the epithelial plane. **(H)** Optical section image crossing the epithelial plane, in which a bacteriocyte-like structure with an external pit is seen. The bacteriocyte-like structures are actually extracellular cavities round in shape, full of tubular bacterial cells, and surrounded by the midgut epithelial cells. **(I–K)** Localization of the symbiont in ovaries and oocytes. **(I)** Localization of the symbiont in the ovarial ampulla located at the interface of lateral oviduct and oocyte-containing ovarioles. **(J)** Optical section image of the ovarial ampulla, in which peculiar host cells are densely populated by the symbiont cells. **(K)** Optical section image of the posterior pole of an oocyte, to which the tubular symbiont cells are infecting through the follicle cell layer. Abbreviations: fc, follicle cell layer; hg, hindgut; mg, midgut; mt, Malpighian tubule; oa, ovarial ampulla; oo, oocyte; ov, ovary; ovd, oviduct; sv, seminal vesicle; ter, tergite; tf, terminal filament. In **(B,C)**, arrowheads indicate the location of the ovarial ampulla. In **(G,H)**, "out" and "in" indicate outside and inside of the midgut, respectively. In **(H)**, an arrow highlights a pit associated with the bacteriocyte-like structure.

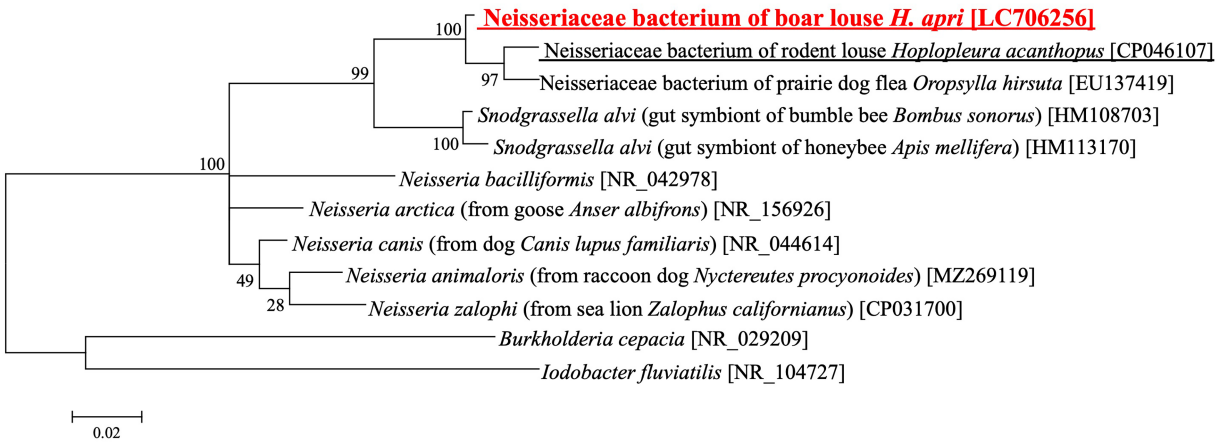

FIGURE 4
Phylogenetic placement of the *Neisseria/Snodgrassella*-allied bacterium of the boar louse *H. apri* in the Neisseriaceae based on 16S rRNA gene sequences. A maximum-likelihood phylogeny inferred from 1,063 aligned nucleotide sites is shown with bootstrap probability at each node (model: HKY+G+I). Host insect information is shown in parentheses, whereas accession number is indicated in brackets. The louse derived sequence determined in this study is highlighted in red, whereas the louse derived sequence reported in a previous study is underlined.

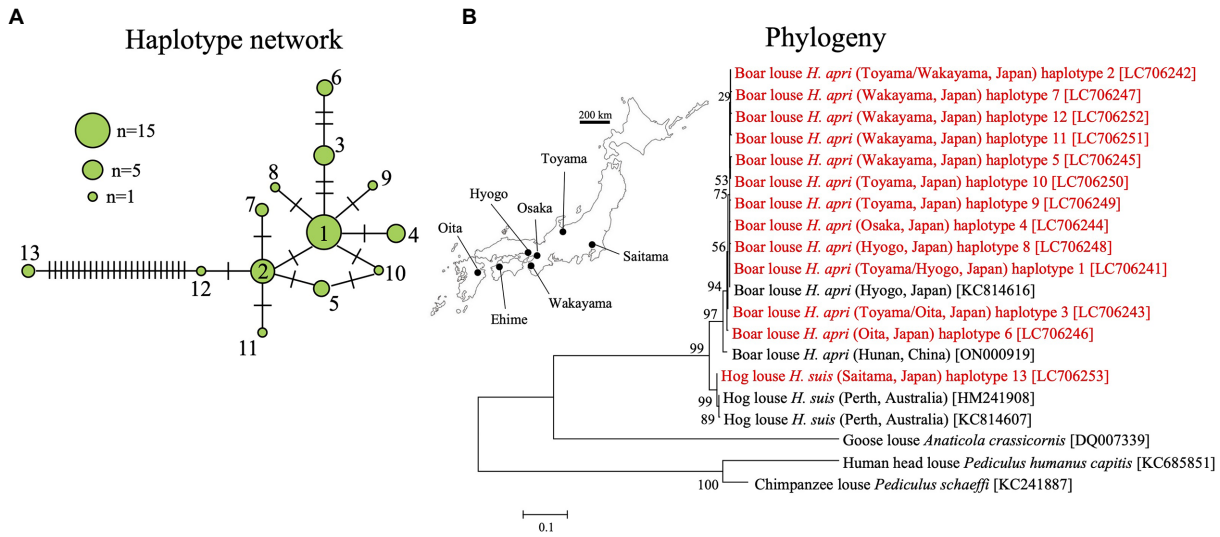

FIGURE 5
(A) Haplotype network of mitochondrial COI gene haplotypes representing 44 individuals from five collection localities of *H. apri* and two individuals from a collection locality of *H. suis*. **(B)** Phylogenetic relationship of the COI haplotypes of *H. apri* and *H. suis*. COI gene sequences of *H. apri* from Japan and China, and those of *H. suis* from Australia, were retrieved from the DNA databases and analyzed together. A maximum-likelihood phylogeny inferred from 516 aligned nucleotide sites is shown with bootstrap probability at each node (model: HKY+G+I). Collection locality information is shown in parentheses, whereas accession number is indicated in brackets. The collection localities are depicted on the map of Japan. The sequences determined in this study are highlighted in red.

populations formed compact and well-supported sister clades, respectively (Figure 5B).

Discussion

In the recognized diversity of the symbiotic organs and the endosymbiotic bacteria among sucking lice (Ries, 1931; Buchner, 1965; Rio et al., 2016; Husnik, 2018), previous studies on the endosymbiotic system in *Haematopinus* ungulate lice were restricted to early histological descriptions on *H. suis* and *H. eurysternus* (Florence, 1924; Ries, 1931; Żelazowska and Biliński, 1999) and a brief molecular phylogenetic characterization for *H. suis*, *H. eurysternus* and *H. apri* (Hypša and Křížek, 2007). As for the boar louse *H. apri*, the only available information on its endosymbiont has been a single 16S rRNA gene sequence (Hypša and Křížek, 2007). In

this study, we investigated the endosymbiotic microbiota of *H. apri* samples derived from multiple local populations using amplicon sequencing, molecular phylogenetic and FISH histological approaches, thereby unequivocally identifying microbiological nature, *in vivo* localization and infection dynamics of the boar louse endosymbiont.

Our amplicon sequencing analysis targeting the hypervariable V3/V4 region of bacterial 16S rRNA gene identified a specific bacterial associate predominantly found from all the local populations of *H. apri* (Figure 1). Molecular phylogenetic analysis based on bacterial 16S rRNA gene sequences showed that the bacterium is closely related to and forming a distinct clade with the endosymbionts of *H. suis*, *H. apri* and *H. eurysternus* whose 16S rRNA gene sequences have been reported previously (Hypša and Křížek, 2007) (Figure 2). Detailed histological observations by FISH (Figure 3) revealed that *in vivo* localization and infection dynamics of the bacterium are concordant with the detailed early histological descriptions on the endosymbiont localization in *H. eurysternus* (Ries, 1931), and, though less comprehensive, in *H. suis* (Florence, 1924; Żelazowska and Biliński, 1999). Therefore, we conclude that this bacterium is the primary endosymbiont of *H. apri*, and suggest that this bacterial clade may represent the primary endosymbiont clade of *Haematopinus* ungulate lice.

Here, however, we point out that the endosymbiont gene sequence of Japanese *H. apri* was considerably different, by 15 nucleotide sites, from that of Czech *H. apri* (Supplementary Figure S2A). Notably, while the endosymbiont gene sequence of Japanese *H. suis* was identical to that of American *H. suis*, the endosymbiont gene sequence of Czech *H. suis* differed by 6 nucleotide sites in comparison with that of Japanese *H. suis* (Supplementary Figure S2A). These patterns may be explained by the following alternative hypotheses: (i) the endosymbionts of Japanese and American *H. apri* and *H. suis* are genetically differentiated from the endosymbionts of European *H. apri* and *H. suis*, respectively; or (ii) the endosymbiont gene sequences of Czech *H. apri* and *H. suis* (Hypša and Křížek, 2007) contain sequencing errors. We note that the endosymbiont sequence of Czech *H. apri* (DQ076665) contains an ambiguous (N) nucleotide site. Collection and analysis of more samples of *H. apri* and *H. suis* from Europe and other regions in the world will clarify which of these hypotheses is more appropriate.

In this study, we found that, on the basis of mitochondrial COI gene sequences, Japanese *H. apri* samples from different local populations are, though with some genetic differences, genetically coherent, whereas they are genetically distinct from Japanese *H. suis* samples (Figure 5A). Molecular phylogenetic analysis of the COI gene sequences, together with those of Chinese *H. apri* and Australian *H. suis* retrieved from the DNA databases, revealed that *H. apri* and *H. suis* are in a sister clade relationship (Figure 5B). However, considering the limited number of samples derived from the limited number of original localities examined in this study, our result should be regarded as tentative and treated with caution, with the following conditions kept in mind.

Thus far, molecular phylogenetic, comparative genomic, and archeological studies have been extensively conducted to clarify how the pig originated from the wild boar *via* domestication. These studies uncovered that (i) domestication of boars into pigs occurred at least twice independently, in East Anatolia and China, about 8,000 years ago, (ii) since then, the domestic pigs spread across the world with human movements, with occasional hybridization with local wild boars, and (iii) therefore, the genomic architecture and the evolutionary trajectory of the domestic pigs are rather mosaic and reticulated (Larson et al., 2005; Frantz et al., 2015, 2016, 2019). In this context, it is of great interest how the boar louse *H. apri*, the hog louse *H. suis*, and their endosymbionts have evolved in the process of swine domestication. The phylogenetic relationship, the species status, and the evolutionary history of *H. apri* and *H. suis* should be established with more louse samples collected from all over the world.

Besides the primary endosymbiont, amplicon sequencing analysis detected relatively minor bacterial associates of *H. apri* and *H. suis*, which were assigned as *Enterobacter*, *Citrobacter*, *Neisseria*, *Streptococcus*, *Serratia*, *Aeromonas*, *Turicibacter*, etc. (Figure 1). On account of the low incidence in the amplicon sequencing data, these bacteria are plausibly facultative and/or casual associates for the host insects. Among them, the *Neisseria*-allied bacterium associated with *H. apri* is of particular interest, because it was recently reported that *Neisseria/Snodgrassella*-allied bacteria are associated with rodent lice of the genera *Hoplopleura* and *Polyplax* as their potential endosymbionts (Říhová et al., 2021). Molecular phylogenetic analysis verified that the *Neisseria*-like bacterium of *H. apri* is closely related to Neisseriaceae bacteria from the rodent louse *H. acanthopus* and the prairie dog flea *Oropsylla hirsute*, and also to the gut symbionts of bees *Snodgrassella alvi* (Figure 4). It is also notable that *Neisseria* species tend to be associated with mammals and birds, such as *N. canis* with dog, *N. animaloris* with racoon dog, *N. zalophi* with sea lion, *N. arctica* with goose, etc. (see Figure 4), as inhabitants of the mucous membranes of animals (Tønjum, 2015). These observations suggest the possibility that, although speculative, the blood-sucking lice may acquire the *Neisseria*-allied bacteria from their host animals either casually or as commensal associates, and some of them have established an obligatory association as observed in *H. acanthopus* (Říhová et al., 2021).

On the basis of the distinct microbiological, phylogenetic and histological features described in this and previous studies, we propose the designation '*Candidatus* Haematopinicola symbiotica' for the hitherto unnamed endosymbiotic bacterial clade associated with ungulate lice of the genus *Haematopinus*. The generic name highlights the endosymbiotic association with *Haematopinus* spp., and the specific name indicates the endosymbiotic nature of the bacterial clade. Thus far, the cattle louse *H. eurysternus* (Ries, 1931; Hypša and Křížek, 2007), the hog louse *H. suis* (Sikora, 1919; Buchner, 1920; Florence, 1924; Ries, 1931; Żelazowska and Biliński, 1999; Hypša and Křížek, 2007; this study) and the boar louse *H. apri* (Hypša and Křížek, 2007; this study) have been shown to host '*Ca.* H. symbiotica'. Histologically, the horse louse *H. asini* seems likely to harbor '*Ca.* H. symbiotica' (Buchner, 1965). Whether the other

Haematopinus species are also associated with '*Ca.* H. symbiotica' requires verification in future studies.

In conclusion, we characterized the primary endosymbiont of the boar louse *H. apri* in detail, uncovered its population genetic and phylogenetic aspects in relation to host's local populations, and proposed the designation '*Ca.* H. symbiotica' for the primary endosymbiont clade of the louse genus *Haematopinus*. The biological role of '*Ca.* H. symbiotica' is expected as provisioning of B vitamins for the blood-sucking host lice, which should be verified by sequencing and analysis of the endosymbiont genomes. Extensive worldwide collection of *H. apri* and *H. suis* samples and genetic analysis of them and their endosymbionts would uncover how the boar louse, the hog louse, and their endosymbionts have evolved in the process of swine domestication in the human history.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

YN performed molecular phylogenetic and amplicon sequencing analyses with support by MaM and MiM. KO conducted histological analyses with support by RK. TF conceived the study and arranged collection of insect samples. YN and TF wrote the paper. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the Japan Science and Technology Agency (JST) ERATO Grant Numbers JPMJER1803 and JPMJER1902 to RK and TF, and by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number JP17H06388 to TF. KO was supported by the JSPS Research Fellowships for Young Scientists Number 21 J01321.

Acknowledgments

We thank Ai Takano, Shigehiko Uni, Takeo Yamauchi and Yuki Miyaoka for providing louse samples.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.962252/full#supplementary-material

SUPPLEMENTARY TABLE S1
Samples of *H. apri* and *H. suis* used in this study with sequence accession numbers.

SUPPLEMENTARY FIGURE S1
FISH detection and RNase digestion control of the primary endosymbiont in the midgut epithelium of *H. apri*.

SUPPLEMENTARY FIGURE S2
(**A**) Nucleotide differences among 16S rRNA gene sequences of the primary endosymbionts of *Haematopinus apri* and *Haematopinus suis* from Japan, United States and Czech. Number of nucleotide differences out of 1100 aligned nucleotide sites is indicated for each of the sequence pairs. Note that the sequence of *H. apri* symbiont Czech [DQ076665] contains an ambiguous (N) site. (**B**) Nucleotide substitutions among mitochondrial COI gene sequences of *H. apri* and *H. suis* from Japan, Australia and China. Number of nucleotide differences out of aligned nucleotide sites is indicated for each of the sequence pairs. The sequences determined in this study are highlighted in red.

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 08 November 2022
DOI 10.3389/fmicb.2022.1031535

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

RECEIVED 30 August 2022
ACCEPTED 11 October 2022
PUBLISHED 08 November 2022

CITATION
Nishide Y, Sugimoto TN, Watanabe K, Egami H and Kageyama D (2022) Genetic variations and microbiome of the poultry red mite *Dermanyssus gallinae*. *Front. Microbiol.* 13:1031535. doi: 10.3389/fmicb.2022.1031535

Genetic variations and microbiome of the poultry red mite *Dermanyssus gallinae*

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The poultry red mite *Dermanyssus gallinae* poses a significant threat to the health of hens and poultry production. A comprehensive understanding of *D. gallinae* is necessary to develop sustainable and efficacious control methods. Here we examined 144 *D. gallinae* collected from 18 poultry farms throughout the Japanese Archipelago for their genetic variations based on mitochondrial cytochrome c oxidase subunit I (COI) sequences, and microbiome variations based on amplicon sequencing of the 16S ribosomal RNA gene. According to COI sequencing, the Japanese samples were categorized into three haplogroups, which did not reflect the geographical distribution. Microbiome analyses found that the major bacteria associated with *D. gallinae* were *Bartonella*, *Cardinium*, *Wolbachia*, and *Tsukamurella*, with *Bartonella* being most predominant. Among 144 individual mites, all possessed one of the two major types of *Bartonella* (*Bartonella* sp. A), while 140 mites possessed the other type (*Bartonella* sp. B). The presence of the two strains of *Bartonella* was also confirmed by a single copy gene, *rpoB*. The presence of *Bartonella* in laid eggs suggested transovarial vertical transmission. Given that obligate blood-feeding arthropods generally require a supply of B vitamins from symbiotic bacteria, *Bartonella* may play an important role in mite survival. *Rickettsiella*, a major symbiont in European *D. gallinae* populations, and suggested to be an important symbiont by genomic data, was rarely found in Japanese populations. *Cardinium* detected from *D. gallinae* fell into a major clade found widely in arthropods, whereas *Wolbachia* detected in Japanese *D. gallinae* appear to be a new lineage, located at the base of *Wolbachia* phylogeny. Of the mitochondrial phylogeny, infection patterns of *Cardinium* and *Wolbachia* were strongly correlated, possibly suggesting one or both of the symbionts induce reproductive manipulations and increase spread in the host populations.

KEYWORDS

symbiont bacteria, *Bartonella*, *Rickettsiella*, *Cardinium*, *Wolbachia*

Introduction

The poultry red mite *Dermanyssus gallinae* (Acari: Dermanyssidae), an obligate blood-feeding ectoparasite that feeds on avian blood, is globally distributed (Chauve, 1998; Sparagano et al., 2009; Wang et al., 2010), and is endemic in many commercial poultry farms, with 80%–90% of egg-laying facilities being infested (Sparagano et al., 2009; George et al., 2015). Once these mites invade a poultry house, their numbers can increase dramatically because typical conditions within poultry houses (high temperature and humidity) are ideal for *D. gallinae*. The densities of *D. gallinae* often reach up to 50,000 mites per bird. In extreme cases when densities reach 500,000 mites per bird, a hen can lose more than 3% of its blood volume every night (Kilpinen et al., 2005). Such heavy mite infestations seriously impact hen health and welfare, resulting in anemia and irritation, and can cause a 10-fold increase in hen mortality (Sigognault Flochlay et al., 2017). Predictably, *D. gallinae* causes a significant reduction in both egg quality and production (Kilpinen et al., 2005; Sparagano et al., 2014). In Europe, *D. gallinae* infestation costs the poultry industry over €231 million annually (Sigognault Flochlay et al., 2017). Furthermore, the prevalence of *D. gallinae* is expected to increase due to increasing acaricide resistance, climate change, and the lack of a sustainable and efficacious approach to control infestations (Chauve, 1998; Nordenfors et al., 2001). Increasing fundamental knowledge of *D. gallinae* can provide insights into new control methods.

Obligatory hematophagy, the practice of feeding exclusively on blood throughout all life stages, is found in a variety of arthropods. Because blood is nutritionally unbalanced, with high levels of protein, iron, and salt, but few carbohydrates, lipids, or vitamins, obligatorily hematophagous arthropods typically rely on symbiotic bacteria to obtain B vitamins (Husnik, 2018; Duron and Gottlieb, 2020). For example, tsetse flies *Glossina* spp. and the bed bug *Cimex lectularius* depend on their endosymbionts *Wigglesworthia glossinidia* and *Wolbachia* for their B vitamin supplies, respectively (Akman et al., 2002; Hosokawa et al., 2010; Michalkova et al., 2014; Nikoh et al., 2014; Moriyama et al., 2015). However, a controversy exists over vitamin-supplying symbiotic bacteria of the obligatory hematophagous *D. gallinae*. Initially, *Rickettsia* was suggested to be a symbiotic bacterium of *D. gallinae* in France using PCR amplification and fingerprinting methods (De Luna et al., 2009; Moro et al., 2009). According to Hubert et al. (2017), however, *Bartonella*-like bacteria rather than *Rickettsia* were considered as the mutualistic symbionts of *D. gallinae* because *Bartonella*-like bacteria was found in four of four sampling sites in Czechia, and in all stadia including eggs by amplicon sequencing. In contrast, another study showed that *Rickettsiella* was widespread in Europe and pseudogenized for many genes, including those involved in the amino acid synthesis pathway, but had an almost full set of genes for B vitamins biosynthesis (Price et al., 2021). In the aforementioned Czech study (Hubert et al., 2017), *Rickettsiella* was found in samples from only one of the four sites. Considering these discrepancies, this study investigated the genetic variations and microbiome variations of *D. galline* in Japanese poultry populations.

Materials and methods

Mite collection

In total, 144 individual *D. gallinae* mites collected from 18 poultry farms in 16 Japanese prefectures were brought to the laboratory alive and preserved in 99.5% ethanol at 4°C until DNA extraction was performed (Figure 1; Supplementary Table 1).

DNA extraction, PCR, and cloning

After being crushed using an EOG-sterilized BioMasher II (Nippi, Inc., Tokyo, Japan), DNA was extracted from the whole body of each adult *D. gallinae* using a DNeasy Blood and Tissue Kit (Qiagen, N.V., Venlo, Netherlands) with 50 µl of EB buffer. The DNA samples were stored at −30°C until used. PCR was conducted for mitochondrial cytochrome *c* oxidase subunit I (COI) using KOD FX Neo (Toyobo Co. Ltd., Osaka, Japan), and the primers FCOIDG and RCOIDG designed for COI of

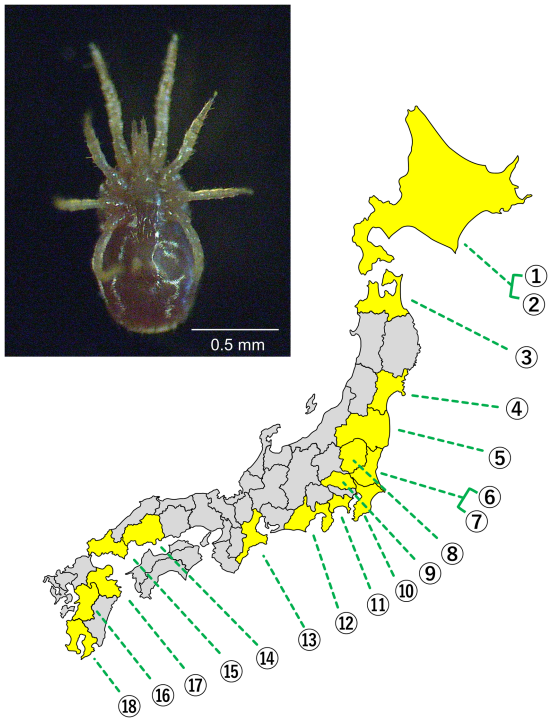

FIGURE 1
(Upper) Ventral view of adult *D. gallinae*. (Lower) A map of the Japanese archipelago. Prefectures where the mites were sampled are depicted in yellow. Each number represents a poultry farm. The exact location of each poultry farm is concealed.

D. gallinae (Øines and Brännström, 2011). For 16S ribosomal RNA sequencing of symbiont bacteria, PCR was conducted using Ex Taq HS (Takara Bio Inc., Kusatsu, Japan) and two sets of universal primers, 10F-1507R or 10FF-1515R. The PCR fragment was cloned using pGEM-T Easy Vector (Promega, Madison, WI, USA), Mighty mix for DNA ligation (Takara), and *Escherichia coli* DH5α competent cells (Takara). Plasmids were extracted using a QIAprep Spin miniprep kit (Qiagen), the purified plasmids were subjected to sequencing reactions using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the sequencing primers M13F or M13RV in the flanking regions of the vector along with the bacterial universal primer 16SA2. The primer sequences are shown in Supplementary Table 2.

Molecular phylogenetic analysis

The molecular phylogenetic analyses were conducted utilizing maximum-likelihood estimation methods using MEGA: Molecular Evolutionary Genetics Analysis, ver. 5.2 software (Tamura et al., 2011) with 1,000 bootstrap replications. The optimum model was selected through model tests to describe each phylogenetic tree. To analyze the genetic diversity of COI sequences, a network diagram of COI haplotypes was drawn using TCS 1.21 software (Templeton et al., 1992).

Amplicon sequencing

To analyze the *D. gallinae* microbiome, hypervariable V3/V4 regions of the 16S rRNA gene were sequenced. The libraries created by using 2-step tailed PCR (Supplementary Table 2) were checked using a Synergy H1 (BioTek, Winooski, VT, USA) and a QuantiFluor dsDNA System (Promega), and the qualities were verified using a Fragment Analyzer (Agilent, Santa Clara, CA, USA) and a dsDNA 915 Reagent Kit (Agilent). These libraries were sequenced using a Miseq sequencer (Illumina, Inc., San Diego, CA USA), and the raw data were deposited in the GenBank sequence database (Accession No. DRR376882–DRR377025).

Bioinformatic analysis of microbiota

The raw amplicon sequences were individually demultiplexed and converted to FASTQ files, which were then analyzed using the Quantitative Insights Into Microbial Ecology (QIIME2) v.2020.06 pipelines (Bolyen et al., 2019). Denoising and quality control, quality filtering, correction of errors in marginal sequences, removal of chimeric sequences, removal of singletons, joining of paired-end reads, and dereplication were conducted using the DADA2 plugin with the following option: -p-trim-left-f 10 -p-trim-left-r 10 -p-trunc-len-f 270 -p-trunc-len-r 270 (Callahan et al., 2016). The DADA2 algorithm was also used to cluster representative amplicon sequence variants and to provide count frequencies for each sample. Taxonomy classification was assigned to the representative amplicon sequence variants based on the Silva v.128 99% operational taxonomic units (OTU) reference sequences[1], and the GreenGenes V.13_8 99% OTU reference sequences[2] databases at 97% OTU level, trained using a Naïve Bayes classifier (classify-sklearn) and the q2-feature-classifier QIIME2 plugin (McDonald et al., 2012; Quast et al., 2013; Bokulich et al., 2018). The resulting relative abundance table of annotated amplicon sequence variants was exported and used to generate taxonomy bar plots in order to visualize the relative abundance of the microbiome using QIIME2 VIEW[3].

Analysis of *Bartonella* on *rpoB* sequences

Two distinct 16S rRNA gene sequences of *Bartonella* (*Bartonella* sp. A and *Bartonella* sp. B) may represent either the existence of distinct bacteria or the intragenomic operon copies of a single bacterium. To distinguish these possibilities, we performed PCR, cloning, and sequencing for the single copy gene *rpoB* from an individual (18_4) whose microbiome was occupied mostly with *Bartonella* sp. A and *Bartonella* sp. B with similar abundance. A primer set, Univ_rpoB_F_deg and Univ_rpoB_R_deg (Supplementary Table 2; Ogier et al., 2019), was used for PCR and the cloning and sequencing were performed as described above.

Diagnostic PCR for *Rickettsiella*

PCR was conducted using KOD FX Neo or Ex Taq HS. The primers Rick-F and Rick-R were the same as those used in a previous study (Price et al., 2021). The amplicon was verified using 1.5% (w/v) agarose/TAE gel.

Confirmation of bacteria in eggs

DNA was extracted from a pool of approximately 20 eggs freshly laid by *D. gallinae* females. The eggs were washed with 0.1% (w/v) benzalkonium chloride followed by two 5 min washes in 70% (v/v) ethanol, and were then subjected to DNA extraction and PCR using 27F-short and 1507R primers (Price et al., 2021). Cloning, plasmid extraction, and sequencing were performed as stated previously.

1 https://data.qiime2.org/2022.2/common/silva-138-99-515-806-nb-classifier.qza

2 https://data.qiime2.org/2022.2/common/gg-13-8-99-515-806-nb-classifier.qza

3 https://view.qiime2.org/

Fluorescence *in situ* hybridization

Whole-mount fluorescent *in situ* hybridization (FISH) targeting bacterial 16S rRNA was performed using the mites collected from Aomori Prefecture following Koga et al. (2009). For the detection of bacterial 16S rRNA, we used the probe EUB338 (5′-GCT GCC TCC CGT AGG AGT-3′) (Amann et al., 1990) labeled with Alexa Fluor 555 at the 5′ terminus. The samples were incubated in hybridization buffer containing 50 nM probe. After being washed in PBST, host nuclear DNA was stained with 4.5 μM 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific). Then, the samples were washed with PBST again, mounted in SlowFade Diamond Antifademountant (Thermo Fisher Scientific), and observed under a laser scanning confocal microscope (LSM 700; Carl Zeiss, Germany).

Statistical analysis

In order to test whether the two bacteria, *Cardinium* and *Wolbachia*, share evolutionary trajectories in maternal lineages, the log marginal likelihood estimated on the COI tree with an assumed independent model was compared with that estimated with an assumed dependent model using BayesTraits V4.0.0.[4] The log Bayes Factor (LBF), twice the difference between the two log marginal likelihoods, was used to interpret whether presence of the two bacteria were correlated (Gilks et al., 1995). LBF < 2 is considered weak evidence, 2 < LBF < 5 is considered positive evidence, 5 < LBF < 10 is considered strong evidence, and LBF >10 is considered very strong evidence.

Results and discussion

Mitochondrial COI sequences of *Dermanyssus gallinae*

By sequencing the mitochondrial COI region of 144 *D. gallinae* mites collected from 18 poultry farms (Figure 1), 31 haplotypes were categorized into three haplogroups AJ1, BJ1, and BJ2 according to previous studies (Øines and Brännström, 2011; Chu et al., 2015; Figure 2; Supplementary Figure 1; Supplementary Table 3). These haplogroups, however, do not necessarily reflect phylogenetically supported clades. The primary haplogroup BJ1 was closely related to several European populations (Norwegian, Swedish, Belgian) and a Korean population, whereas BJ2 was related to a Czech population (Figure 2; Supplementary Figure 2; Supplementary Table 4). These results may represent inter-country transport of contaminated material or infested birds as previously suggested (Øines and Brännström, 2011; Chu et al., 2015). AJ1 has only been found in the Japanese population, although it is relatively similar to the French population. AJ2 was found in Japan approximately 10 years ago (Chu et al., 2015), but was not found in the present study. Because multiple haplogroups were sometimes seen on a single poultry farm (Supplementary Table 3), invasions of *D. gallinae* are not likely to be very rare. In each poultry farm, chicken breeds are selected depending on their purpose (whether for eggs or meat), but mitochondrial haplogroups of *D. gallinae* were not associated with chicken breeds (purposes) (Supplementary Table 1). All 144 sample mites examined in the present study were identified as members of the well-supported clade of *D. gallinae*. Note that, as written below, *D. gallinae* possesses *Wolbachia* and *Cardinium*, which may confound the inference of an arthropod's evolutionary history from mtDNA data because of maternal inheritance and linkage disequilibrium with mitochondria. Finally, the northern fowl mite, *Ornithonyssus sylviarum*, which resembles *D. gallinae* in size and appearance (Nakamae et al., 1997; Di Palma et al., 2012), is also known as a serious pest in poultry farms, but *O. sylviarum* was not found in the present samples.

Microbiome of *Dermanyssus gallinae*

For the collected 144 sample individuals, microbiomes were analyzed through amplicon sequencing of the hypervariable V3/V4 region of 16S rRNA; 8,939,852 reads were reduced into 819 OTU (Supplementary Table 5). The 11 major OTU were *Bartonella* sp. B, *Cardinium*, *Bartonella* sp. A, *Wolbachia*, *Tsukamurella*, *Micrococcus*, *Rickettsiella*, *Staphylococcus*, *Enterobacter*, *Rhodopseudomonas,* and *Psychrobacter* in order of relative frequency (Figure 3; Supplementary Table 6). By assuming that an individual mite has the bacterium when the bacterium represented more than 1% of the tags analyzed, we showed that among the 144 individual mites, all had *Bartonella* sp. A (100%), 140 mites had *Bartonella* sp. B (97.2%), 76 mites had *Cardinium* (52.8%), 52 mites had *Wolbachia* (36.1%), 23 mites had *Tsukamurella* (16.0%), 17 mites had *Micrococcus* (11.8%), and 2 mites had *Rickettsiella* (1.4%). Box plots of the 18 poultry farms were drawn according to the Shannon diversity index (Supplementary Figure 3; Supplementary Table 7).

While *Rickettsia* was previously suggested to be a symbiotic bacterium of *D. gallinae* (De Luna et al., 2009; Moro et al., 2009), the reads of Rickettsiales were not found in the present study, except for *Wolbachia* and a very few reads of plant mitochondria. Additionally, *Spiroplasma*, which was previously detected in a French *D. gallinae* study (Moro et al., 2009), was not found in the present study. A few reads found in some samples were *Erysipelothrix* and *Pasteurella*, which are suspected hen and human pathogens which have been detected in *D. gallinae* (Chirico et al., 2003; Moro et al., 2009; Eriksson et al., 2010; Moro et al., 2011; Hubert et al., 2017). However, the *Erysipelothrix* sequences obtained in this study had only 82–87% similarity with known hen-pathogenic *Erysipelothrix rhusiopathiae* sequences (Takahashi et al., 1994; Accession No.: NR_040837), whereas the *Pasteurella* sequences obtained in this study had 93%–94% similarity with known human opportunistic pathogen *Pasteurella*

4 http://www.evolution.reading.ac.uk/BayesTraitsV4.0.0/BayesTraitsV4.0.0.html

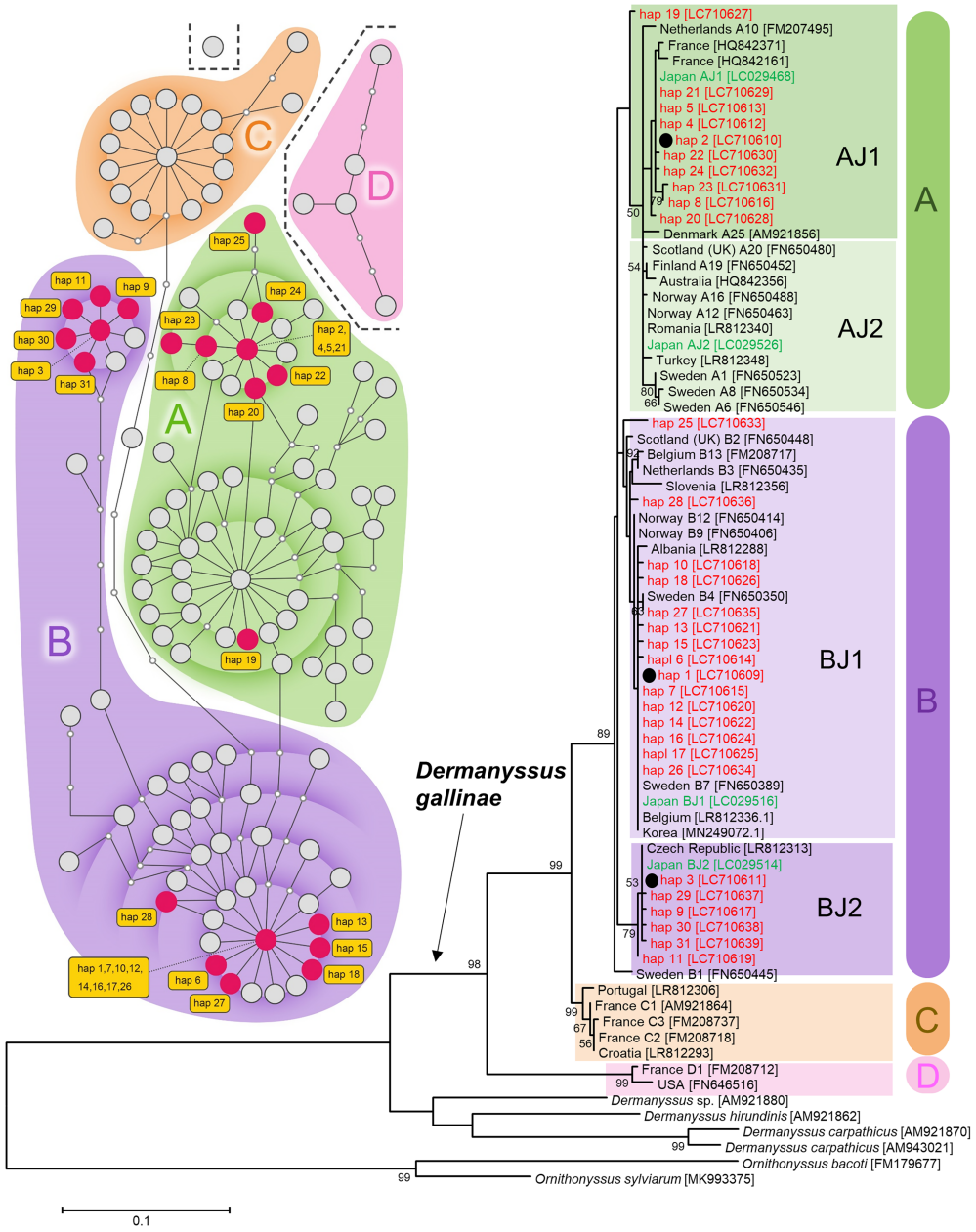

FIGURE 2
Mitochondrial DNA diversity of Japanese *D. gallinae* examined in this study among global samples of *D. gallinae*. *Left*: A TCS haplotype network based on COI sequences (314bp; 122 OTU) of *D. gallinae*. *Right*: A maximum-likelihood phylogenetic tree (model T92+G) based on COI sequences (469bp; 75 OTU) of *D. gallinae* together with three other species of *Dermanyssus* and two species of *Ornithonyssus*. Based on the 469-bp sequences, 144 individual mites examined in this study fell into 31 haplotypes (hap 1–hap 31). Haplogroups **(A–D)** are shaded by respective colors. For the network, existing haplotypes are represented by large circles, and missing haplotypes are represented by small circles which are connected by branches representing 1-bp substitutions. Based on the 314-bp sequences, the 31 haplotypes were assigned into 21 haplotypes (indicated by red circles in the network). For the phylogenetic tree, the three most representative haplotypes (hap 1, hap 2, and hap 3) are indicated with black circles and their bootstrap probability is shown at each node. Nomenclature of haplotypes used in the previous studies (Øines and Brännström, 2011; Chu et al., 2015) are given following the country names. Haplotypes of Japanese samples are shown in red (this study) and green (Chu et al., 2015). Numbers in square brackets indicate accession numbers of NCBI. See Supplementary Table 3 for sample information.

multocida sequences (Kuhnert et al., 2000; Accession No.: NR_041809). Determining whether these *Erysipelothrix* and *Pasteurella* in *D. gallinae* could be pathogenic for hens and/or humans requires further investigation.

Phylogenetic analysis of *Bartonella*

The cloning and sequencing of nearly full sequences of 16S rRNA from one sample (8_1) from Tochigi Prefecture identified

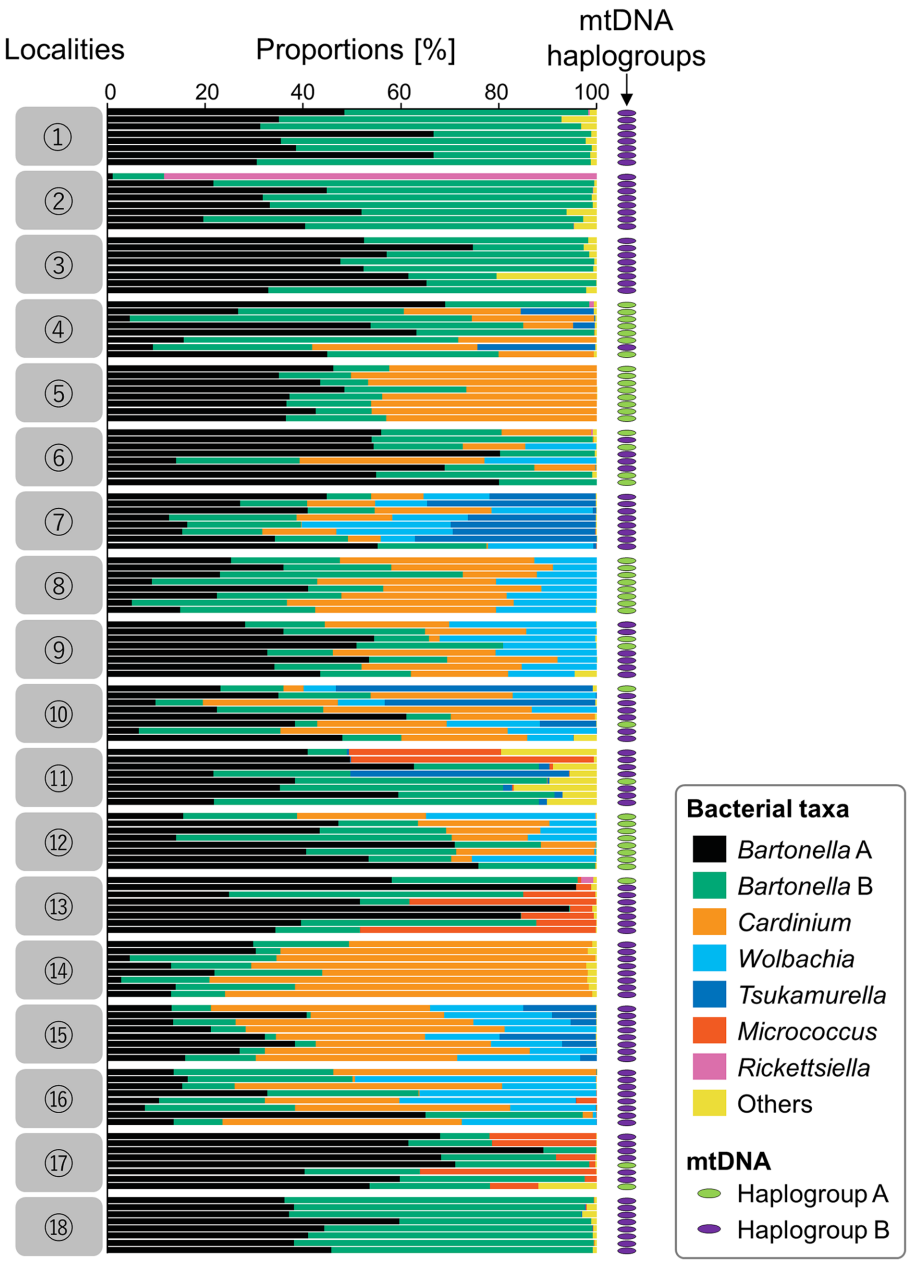

FIGURE 3
Results of amplicon sequencing of the hypervariable V3/V4 region using 144 individual *D. gallinae* mites from 18 poultry farms (eight individual mites from each poultry farm). For each individual, an mtDNA haplogroup (either A or B) is shown by a colored ellipse at the right (green for haplogroup A; purple for haplogroup B). Assigned bacterial taxa are color-coded as shown in the box on the right. Numbers on the left represent geographic localities shown in Figure 1.

two strains of *Bartonella*, with each corresponding to either *Bartonella* sp. A or *Bartonella* sp. B. These sequences were closely related but differed in 66 bp among 1,437 bp, and formed a cluster with the *Bartonella* sp. sequences obtained from *D. gallinae* in a previous study (Hubert et al., 2017; Figure 4A). Although not well-supported by bootstrap value, this cluster was distinct from other *Bartonella* and *Rhizobiales* bacteria. It has been reported that 16S rRNA is not a suitable marker for *Bartonella* (La Scola et al., 2003) because of the existence of multiple copies in its genome (Viezens and Arvand, 2008; Banerjee et al., 2020). To distinguish whether *Bartonella* sp. A and *Bartonella* sp. B described by 16S rRNA amplicon sequencing are actually from a single strain, we sequenced a single copy gene *rpoB* (385 bp). An individual 18_4, whose microbiome was mostly occupied by *Bartonella* sp. A and *Bartonella* sp. B with a nearly equal ratio, was subjected to PCR, cloning, and sequencing (Supplementary Figure 4). All the obtained sequences (17 out of 17) were *Bartonella*-like, which consist of two types of sequences differing in 76 bp. This result strongly suggests that

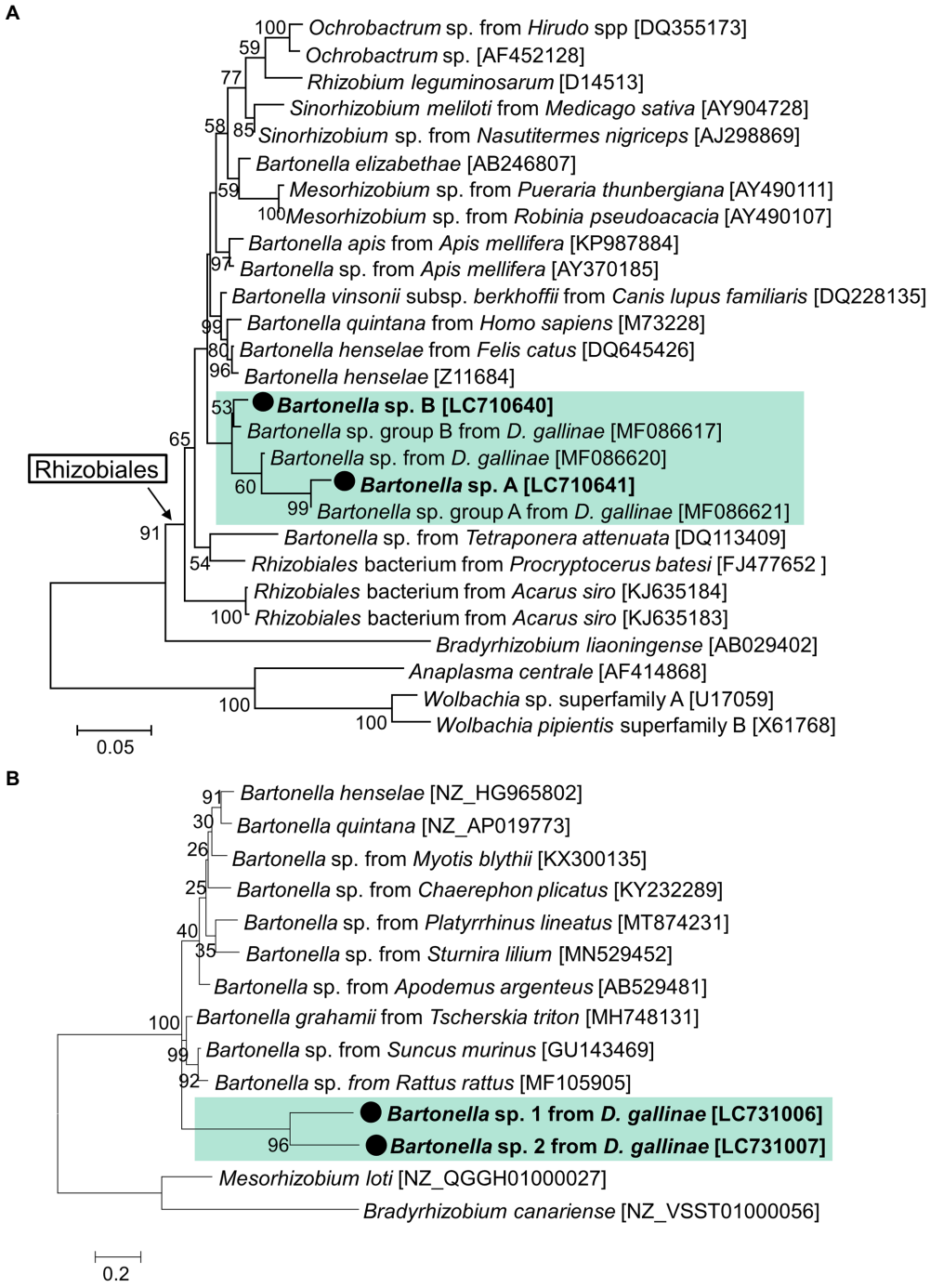

FIGURE 4
Phylogenetic relationship of the 16S rRNA sequences (A) and rpoB (B) of Bartonella and related species. **(A)** A maximum-likelihood phylogeny (model K2+G+I) inferred from 1,175 aligned nucleotide sites are shown with bootstrap probability at each node. **(B)** A maximum-likelihood phylogeny (model K2+G) inferred from 385 aligned nucleotide site. The sequences in bold with black circle are those obtained in this study. OTU of *Bartonella* from *D. gallinae* are shaded with light green. Numbers in square brackets indicate NCBI accession numbers.

Bartonella sp. A and *Bartonella* sp. B are distinct strains of *D. gallinae*. Furthermore, these sequences of *rpoB* were a new lineage that differed from the known *Bartonella* and *Rhizobiales* (Figure 4B). It remains unknown, however, which of the *rpoB* sequences corresponds to *Bartonella* sp. A or *Bartonella* sp. B.

Phylogenetic analysis of *Rickettsiella*

A nearly full sequence of *Rickettsiella* 16S rRNA was obtained from a sample from Hokkaido (2_1) which had a relatively high abundance of *Rickettsiella* in the amplicon

sequencing (Figure 3). This sequence was identical to the *Rickettsiella* sequence obtained from UK samples, and differed by 1 bp from Czech samples (Figure 5). These *D. gallinae Rickettsiella* sequences were related to *Rickettsiella* which infect the relict tick *Haemaphysalis concinna* and the seabird tick *Ixodes uriae*. The clade composed of the mite/tick *Rickettsiella* sequences also include *Rickettsiella* of the pea aphid *Acyrthosiphon pisum* and is distinct from the other clade composed of *Rickettsiella* from insects and isopods.

Probable obligate symbionts: *Bartonella* or *Rickettsiella*?

We hypothesize that *Bartonella* sp. A is necessary for the survival or reproduction of *D. gallinae* because all 144 *D. gallinae* individuals examined had *Bartonella* sp. A. Although, *Bartonella* sp. B might be derived from the blood meal because the 16S rRNA sequence of *Bartonella* sp. B, which was possessed by 140 out of 144 *D. gallinae* individuals (97.2%), matched the sequence of *Bartonella* obtained from sampled avian blood (Accession No. MN320519, MN320520, MN320527). Cloning and sequencing of the bacterial 16S rRNA sequences using surface-sterilized *D. gallinae* eggs from Ibaraki Prefecture (derived from a different farm from those used for amplicon sequencing) identified both *Bartonella* sp. A and B (Figure 6), which is consistent with a previous study which detected *Bartonella* on eggs using amplicon sequencing (Hubert et al., 2017). These results may suggest that both *Bartonella* sp. A and B are vertically transmitted transovarially *via* egg cytoplasm. Then we performed whole-mount FISH targeting bacterial 16S rRNA to examine the localization of symbiotic bacteria using a *D. gallinae* adult and egg collected from Aomori Prefecture. As is shown by microbiome data (population No. 3 in Figure 3), this population has only *Bartonella* sp. A and *Bartonella* sp. B as major bacteria. The FISH preparation showed that, in the adult female, reddish fluorescence is observed throughout the cell cytoplasm except for the nucleus (Supplementary Figure 5), which is typical for intracellular symbionts. The bacteria appear to be present throughout the mite body rather than localized in a distinct bacteriocyte. FISH preparation was also made on eggs, but no clear bacterial image was observed. This may be due to the chorion and/or yolk proteins that inhibit the transmission of fluorescent signals. Note that these results were obtained with the universal 16S rRNA probe for bacteria, so further analysis with *Bartonella*-specific probes would be needed.

Amplicon sequence analysis detected *Rickettsiella* from only 2 out of 144 *D. gallinae* individuals. To test whether *Rickettsiella* was failed to be detected in many samples due to primer mismatches or other factors specific to amplicon sequencing, diagnostic PCR was performed for *Rickettsiella* utilizing primers used in a previous study (Price et al., 2021).

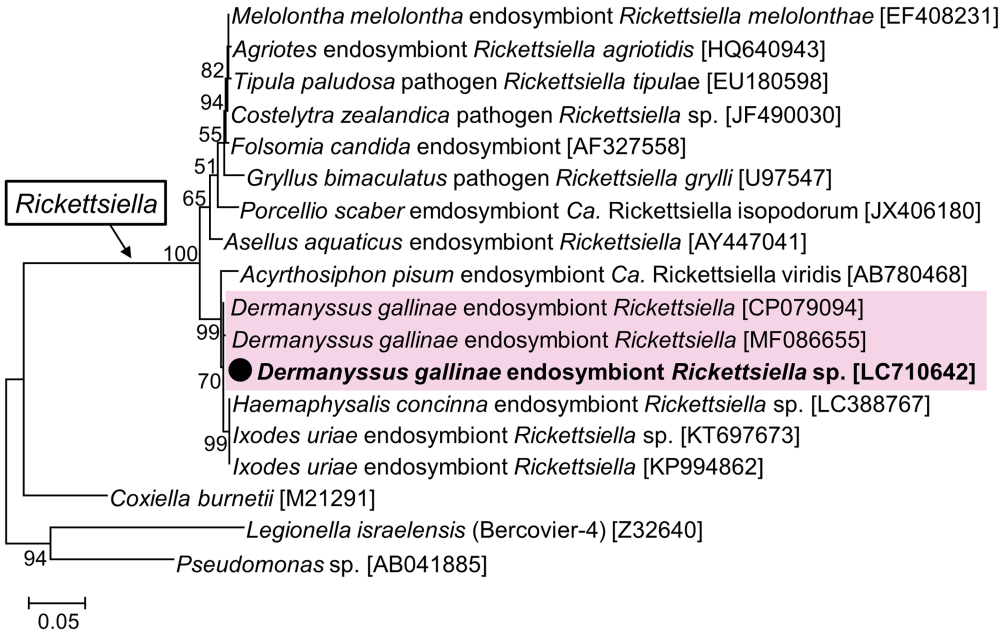

FIGURE 5
Phylogenetic relationship of the 16S rRNA sequences of *Rickettsiella* and related species. A maximum-likelihood phylogeny (model HKY+G+I) inferred from 1,018 aligned nucleotide sites is shown with bootstrap probability at each node. The sequences in bold with black circle are those obtained in this study. OTU of *Rickettsiella* from *D. gallinae* are shaded in pink. Numbers in square brackets indicate NCBI accession numbers.

Among 16 samples from Hokkaido Prefecture (populations No. 1 and 2), *Rickettsiella* was detected from only one individual (2_1), which is consistent with the results of the amplicon sequence analysis (Supplementary Figure 6); we therefore conclude that *Rickettsiella* infection is very rare in Japanese *D. gallinae*. This contrasts with European populations of *D. gallinae*, which have been reported to be highly infected with *Rickettsiella* (De Luna et al., 2009; Moro et al., 2009; Price et al., 2021). In Czechia, however, *Rickettsiella* was detected from only one of four sample sites studied (Hubert et al., 2017). Although no mitochondrial haplotypes were examined in relation to symbiotic bacteria in the European studies, we cannot rule out the possibility that haplogroups A and B have *Bartonella* as an essential symbiont and haplogroup C has *Rickettsiella* as an essential symbiont. This hypothesis may be worth testing in future research.

To summarize, Japanese *D. gallinae* are predominantly infested with *Bartonella*, which likely plays an important role in the survival of these mites. *Bartonella* bacteria generally have a hemotropic lifestyle and is found in mammalian hosts and blood-sucking parasitic arthropods, such as lice and fleas (Kosoy et al., 2012; Gutiérrez et al., 2014; Theonest et al., 2019). We speculate that *Bartonella* contributes to the synthesis of B vitamins, although this has not yet been demonstrated in any hosts.

As noted in the introduction, *Rickettsiella* is considered to be an important symbiont of *D. gallinae* because it has a nearly complete set of B vitamin synthetic pathways (Price et al., 2021). However, the biotin (vitamin B7) synthesis pathway has been shown to be incomplete due to the loss of bioH (Price et al., 2021). In the present study, two *Rickettsiella*-possessing individuals were found that had both *Bartonella* sp. A and B. Considering the contrasting frequencies of *Bartonella* and *Rickettsiella*, it is likely that *Bartonella* is an important symbiont for *D. gallinae*, whereas *Rickettsiella* has an auxiliary role or pathogenic effect, at least in Japanese red mite populations.

Facultative symbionts: *Cardinium* and *Wolbachia*

The nearly full sequences of 16S rRNA of *Cardinium* and *Wolbachia* were obtained from one sample (8_1) from Tochigi Prefecture. Phylogenetically, the *Cardinium* derived from *D. gallinae* fell into the group A of *Cardinium*, the most common group found among various arthropods (Nakamura et al., 2009) (Figure 7A). In contrast, the *Wolbachia* obtained from *D. gallinae* was idiosyncratic, and was located basally of all previously published *Wolbachia*, but formed a well-supported clade with other *Wolbachia* (Figure 7B).

Both *Cardinium* and *Wolbachia* are known as maternally inherited symbionts which manipulate host reproduction in various manners (such as cytoplasmic incompatibility, feminization, male killing, and induction of parthenogenesis)

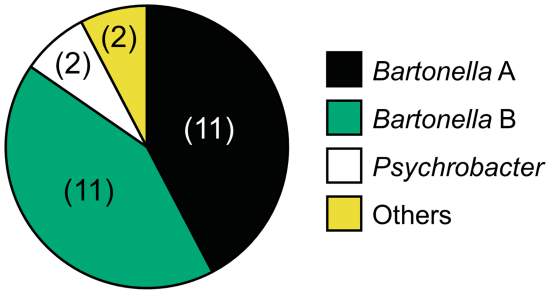

FIGURE 6
Relative abundance of bacteria associated with *D. gallinae* eggs derived from Ibaraki Prefecture based on 16S rRNA sequences obtained by cloning and sequencing of universal PCR products. The three primary bacterial taxa are colored as shown on the right. Two clones (*Staphylococcus* and *Clostridium*) are indicated with yellow. The number of clones (among 26 clones) is shown in parenthesis.

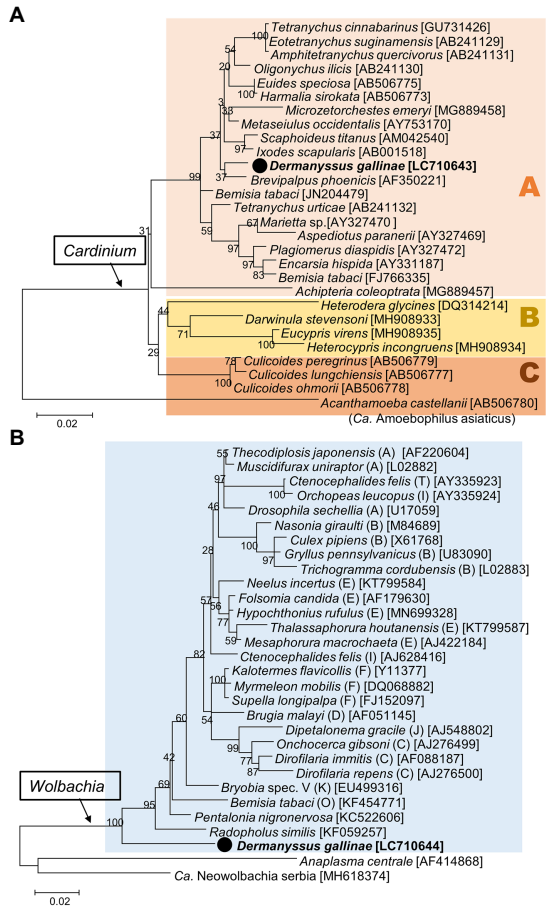

FIGURE 7
Phylogenetic relationships of the 16S rRNA sequences of *Cardinium* **(A)** and *Wolbachia* **(B)**. **(A)** Maximum-likelihood tree (the model is K2+G+I based on 1,175bp) of *Cardinium*, which can be separated into three groups (A, B, and C). **(B)** Maximum-likelihood tree (the model is HKY+G+I based on 1,226bp) of *Wolbachia*. Supergroups of *Wolbachia* are indicated in round brackets. A bootstrap probability is given at each node. The sequences in bold with black circle are those obtained in this study. Numbers in square brackets indicate NCBI accession numbers.

to enhance their own transmission (Werren et al., 2008; Hurst and Frost, 2015). In spider mites, it has been reported that *Wolbachia* and *Cardinium* cause cytoplasmic incompatibility (Breeuwer, 1997; Gotoh et al., 2007), and that *Cardinium* causes feminization (Weeks et al., 2001; Chigira and Miura, 2005). Because of the linkage disequilibrium between maternally inherited bacteria and host mitochondrial DNA, the spread of maternally inherited symbionts can result in indirect selection on mitochondrial DNA (Hurst and Jiggins, 2005). This study compared the infection frequencies of *Cardinium* and *Wolbachia* in respective mtDNA haplotypes (Supplementary Figure 7). While haplogroup BJ1 had a low *Cardinum* and/or *Wolbachia* infection rate (infection rate of either bacterium is 25.4% (18/71)), haplogroup AJ1 and haplogroup BJ2 had very high infection rates of 79.1% (34/43) and 96.7% (29/30), respectively. These conspicuously biased infection rates may indicate that *Cardinum* and/or *Wolbachia* manipulate host reproduction. The LBF that inferred dependency between *Cardinium* and *Wolbachia* was 26.539, which is very strong evidence for correlation (Gilks et al., 1995). We hypothesize that *Wolbachia*, *Cardinium*, or both may induce reproductive manipulations in *D. gallinae* and its symbionts, and that the associated cytoplasms (i.e., mitochondrial haplotypes) spread in the host populations.

Conclusion

We investigated the microbiome of the red poultry mite *D. gallinae* in Japanese populations. Using Illumina Miseq amplicon sequencing, and full 16S rRNA sequencing using universal primers, we identified the following properties of the *D. gallinae* microbiome. First, all individual mites had *Bartonella* sp. A, and most individuals had *Bartonella* sp. B, which are both closely related to the *Bartonella* harbored by *D. gallinae* in Czechia. These *Bartonella* species, which are probably vertically transmitted through eggs, may play important roles in *D. gallinae* survival. Second, unlike many European populations, *Rickettsiella* was rarely found and is unlikely to play an important role in the survival of *D. gallinae*, at least in Japanese populations. Third, *Cardinium* and *Wolbachia* were found at relatively high frequencies, and while the *Cardinium* identified belonged to a lineage commonly found in insects and other arthropods, the *Wolbachia* belonged to a novel lineage that is located basally to all other *Wolbachia* found so far. It is possible that these *Cardinium* and *Wolbachia* strongly impact the mitochondrial genome dynamics of *D. gallinae*. It should also be noted that regarding all the detected bacteria, no direct evidence for their roles within *D. gallinae* was identified because *D. gallinae* could not be successfully reared despite multiple attempts following Bruneau et al. (2001). Inoculation and removal of each bacterium to fulfill Koch's postulate, as well as genome sequencing of each bacterium will be necessary to evaluate the impact of the bacteria on *D. gallinae*.

Data availability statement

The data presented in the study are deposited in NCBI, accession numbers LC710609-LC710644, LC731006-LC731007, and DRR376882–DRR377025.

Author contributions

YN, TS, KW, HE, and DK conceived and designed the experiments. YN organized all the samples and performed experiments. TS analyzed the raw reads of Illumina Miseq. YN wrote the draft manuscript and DK revised the manuscript with the inputs from all authors. All authors contributed to the article and approved the submitted version.

Funding

The authors declare that this study received funding from SC Environmental Science Co., Ltd. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

Acknowledgments

We thank Takayo Nakakura, Masae Takashima, and Maria Murakami for their technical assistance, and Takuhiko Yokoyama for graphical illustration.

Conflict of interest

HE was employed by the company SC Environmental Science Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1031535/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 10 January 2023
DOI 10.3389/fmicb.2022.1050063

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

RECEIVED 21 September 2022
ACCEPTED 16 December 2022
PUBLISHED 10 January 2023

CITATION
Militzer N, Pinecki Socias S and Nijhof AM (2023) Changes in the *Ixodes ricinus* microbiome associated with artificial tick feeding. *Front. Microbiol.* 13:1050063. doi: 10.3389/fmicb.2022.1050063

Changes in the *Ixodes ricinus* microbiome associated with artificial tick feeding

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Artificial tick feeding systems (ATFS) can be used to study tick biology and tick-pathogen interactions. Due to the long feeding duration of hard ticks, antibiotics are commonly added to the *in vitro* blood meal to prevent the blood from decaying. This may affect the ticks' microbiome, including mutualistic bacteria that play an important role in tick biology. This effect was examined by the consecutive feeding of *Ixodes ricinus* larvae, nymphs, and adults *in vitro* with and without the supplementation of gentamicin and in parallel on calves. DNA extracted from unfed females was analyzed by 16S rRNA sequencing. The abundance of *Candidatus Midichloria mitochondrii*, *Rickettsia helvetica* and *Spiroplasma* spp. was measured by qPCR in unfed larvae, nymphs, and adults. Larvae and nymphs fed on calves performed significantly better compared to both *in vitro* groups. Adults fed on blood supplemented with gentamicin and B vitamins had a higher detachment proportion and weight compared to the group fed with B vitamins but without gentamicin. The detachment proportion and weights of females did not differ significantly between ticks fed on calves and *in vitro* with gentamicin, but the fecundity was significantly higher in ticks fed on calves. 16S rRNA sequencing showed a higher microbiome species richness in ticks fed on calves compared to ticks fed *in vitro*. A shift in microbiome composition, with *Ca*. *Midichloria mitochondrii* as dominant species in females fed as juveniles on calves and *R*. *helvetica* as the most abundant species in females previously fed *in vitro* was observed. Females fed *in vitro* without gentamicin showed significant lower loads of *Ca*. M. mitochondrii compared to females fed *in vitro* with gentamicin and ticks fed on calves. *Spiroplasma* spp. were exclusively detected in female ticks fed on cattle by qPCR, but 16S rRNA sequencing results also showed a low abundance in *in vitro* females exposed to gentamicin. In conclusion, the employed feeding method and gentamicin supplementation affected the ticks' microbiome composition and fecundity. Since these changes may have an impact on tick biology and vector competence, they should be taken into account in studies employing ATFS.

KEYWORDS

***Ixodes ricinus*, *Midichloria*, *Rickettsia helvetica*, *Spiroplasma*, *in vitro* feeding, artificial feeding, vitamin B**

1. Introduction

Artificial tick feeding systems (ATFS) in which ticks are fed on artificial membranes or animal skin *in vitro* have been widely used, for instance in studies on tick biology, tick-pathogen interactions and the screening of anti-tick compounds under controlled laboratory conditions (Waladde et al., 1979; Kuhnert et al., 1995; Krober and Guerin, 2007; Antunes et al., 2014; Król et al., 2021; Militzer et al., 2021). In addition, ATFS contribute to the 3R principle to Reduce, Refine and Replace animal experimentation in science. Membrane-based ATFS typically consist of a tick containment unit, a membrane which mimics the skin, a blood meal and a heating device to warm the blood to 37–39°C (Nijhof and Tyson, 2018).

Hard ticks have a long feeding duration of several days to weeks and this period is usually extended when ticks are fed *in vitro* (Kuhnert et al., 1995; Militzer et al., 2021). This makes the use of ATFS challenging as the blood meal may decay due to microbial growth. The blood meal is therefore regularly changed and routinely treated with antimicrobials such as penicillin, streptomycin, rifampicin, phosphomicin, ciprofloxacin and gentamicin, or antimycotic substances such as amphotericin b or nystatin to prevent the blood from decaying (Kemp et al., 1975; Kuhnert et al., 1995; Krober and Guerin, 2007; Oliver et al., 2021). It is plausible that the addition of antimicrobial compounds to the blood meal will also affect the tick microbiome composition, but little is known about the extent of this effect.

It was previously shown that a dysbiosis of the ixodid tick microbiome after injection of ticks with antibiotics resulted in reduced fecundity, feeding, survival and/or development (Zhong et al., 2007; Kurlovs et al., 2014; Guizzo et al., 2017; Li et al., 2018; Ben-Yosef et al., 2020; Zhong et al., 2021). Alternative antibiotic treatment methods, such as feeding ticks on antibiotic-treated animals were also reported to have a negative effect on tick fecundity (Clayton et al., 2015; Zhang et al., 2017; Duron et al., 2018). Dysbiosis could also be induced by wash procedures or sterile maintenance (Narasimhan et al., 2014; Hamilton et al., 2021; Hurry et al., 2021). To our knowledge, only a single study has so far described the dysbiosis of hard ticks by feeding *Ixodes scapularis* female ticks on blood treated with antibiotics through an artificial membrane (Oliver et al., 2021). Changes to the tick microbiome were also shown to affect the vector competence of ticks: dysbiosed *I. scapularis* larvae were for instance less prone to *Borrelia* colonization (Narasimhan et al., 2014), whereas *A. phagocytophilum* colonization was increased in *I. scapularis* nymphs fed on gentamicin-treated mice infected with *A. phagocytophilum* (Abraham et al., 2017).

In Europe, *Ixodes ricinus* is the most widely distributed tick in Europe and the main vector for tick-borne pathogens causing Lyme Borreliosis and Tick-Borne Encephalitis in humans. Like all hard ticks, *I. ricinus* requires a blood meal in each parasitic life stage, i.e., as larvae, nymph and adult, in order to develop and reproduce. In *I. ricinus*, *Candidatus* M. mitochondrii (hereafter *M. mitochondrii*) is the most commonly reported maternally inherited symbiont (Gofton et al., 2015; Aivelo et al., 2019). Other bacteria associated with *I. ricinus* are *Rickettsiella* spp., *Borrelia* spp., *Spiroplasma*, *Rickettsia* spp., *A. phagocytophilum*, and *Candidatus* Neoehrlichia (van Overbeek et al., 2008; Gofton et al., 2015; Aivelo et al., 2019; Garcia-Vozmediano et al., 2021; Lejal et al., 2021). Maternally inherited bacterial endosymbionts play an important role in nutrition, defense and immune pathways. As the blood meal is lacking B vitamins and co-enzymes, it has been suggested that these and other nutrients could be provided to the tick by their endosymbionts (Gottlieb et al., 2015; Smith et al., 2015; Duron et al., 2018; Duron and Gottlieb, 2020). Biosynthesis pathways for certain B vitamins and cofactors were shown to be present in the genomes of some endosymbionts, including *M. mitochondrii* (Duron et al., 2017; Olivieri et al., 2019; Buysse et al., 2021).

The objective of this study was to compare the *in vitro* feeding of *I. ricinus* on bovine blood using an ATFS to a control group of *I. ricinus* ticks fed on cattle (C). To assess the influence of antibiotic treatment on tick feeding parameters in the ATFS, we compared ticks fed *in vitro* on gentamicin-treated blood (IVG^+) to ticks fed *in vitro* on blood without antibiotics (IVG^-). 16S rRNA sequencing was used to identify microbial communities in the ticks. The abundance of three of the most common species present in unfed females: *Rickettsia helvetica*, *M. mitochondrii* and *Spiroplasma* spp. was subsequently quantified by qPCR for samples from unfed larvae, nymphs, females and males.

2. Materials and methods

2.1. Ticks and the *in vivo* feeding

All *I. ricinus* ticks originated from a laboratory colony of the Institute for Parasitology and Tropical Veterinary Medicine of the Freie Universität Berlin. The feeding of each life stage was done in parallel for the IVG^-, IVG^+ and C groups. The study started with the feeding of *I. ricinus* F_0-larvae at 2–5 months post hatching in May–June 2019. The F_0-larvae were the offspring of four females from the laboratory colony. Nymphs that molted from these larvae were fed at 3–4 months post molting between October and November 2019, while the resulting adults were fed between 2 and 8 months after molting between July and September 2020. Ticks of the C group were fed on the ears of tick-naïve Holstein-Friesian calves that were 3.5–4.5 months of age. The estimated number of unfed larvae used was calculated by dividing the weight of the larvae batch by the calculated mean weight of a single unfed larva measured by an analytic scale. For the feeding, the base of each ear was covered with fabric-based tape (Leukoplast, BSN medical, Hamburg, Germany) after which linen ear bags containing equal amounts of ticks were placed over the ears. The ear bags were subsequently attached to the tape at the base of the ear by a second piece of Leukoplast. Detached ticks were collected twice daily. All animal experiments were approved by the regional authority for animal experimentation (LaGeSo, Berlin, 0387/17).

2.2. *In vitro* feeding (IVG$^+$ and IVG$^-$)

All feeding experiments were performed as previously reported (Militzer et al., 2021). Aseptically withdrawn heparinized bovine blood was supplemented with 2 g/L glucose and 0.1 M adenosine triphosphate (ATP, Carl Roth). Due to previous experiences with the artificial feeding of *I. ricinus* adults with blood supplemented with gentamicin, such as long feeding durations and the observation that some attached ticks turned black and died, we decided to supplement the blood meals for adults of both groups (IVG$^+$ and IVG$^-$) with B vitamin components (Militzer et al., 2021). For the ticks fed on blood supplemented with antibiotics (IVG$^+$), 5 µg gentamicin (Cellupur, Carl Roth, Karlsruhe, Germany) was added per mL of blood.

After feeding to repletion, larvae and nymphs of all groups (IVG$^+$, IVG$^-$, and C) were stored at room temperature (RT) and > 90% relative humidity (RH) under a natural light–dark regime. Adult ticks were stored at 20°C, >90% RH in darkness.

2.3. Sample preparation and DNA extraction

Immediately before each feeding experiment, unfed tick samples were collected and stored at −20°C. Prior to DNA extraction, all unfed ticks were surface-sterilized as previously described (Binetruy et al., 2019b). This was performed by washing the ticks in 1% commercial bleach for 30 s, followed by a rinsing for 1 min in three successive baths of DNA-free water. Individual females were quadrisected and nymphs and males were bisected to facilitate subsequent homogenization by crushing with a pestle. Only sterile tubes, scalpel blades and pestles were used. Genomic DNA extraction was performed using the Nucleospin Tissue XS kit (Macherey-Nagel) following the manufacturer's protocol, with an overnight lysis step and a final elution volume of 40 µL. All eluates were evaporated at RT for 10 min to remove residual ethanol. Extraction was performed for individual nymph and adult samples ($n = 5–8$ per experimental group) or in batches for larvae ($n = 13–20$). Negative controls were included for each batch of extracted DNA and consisted of tubes without tick material that were processed together with the tick samples.

2.4. Bacterial 16S rRNA sequencing

DNA from unfed ticks and negative controls were used for further NGS analysis. To amplify a 466 bp fragment spanning the V3-V4 region of bacterial 16S rRNA, primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNN GGGTATCTAAT-3′) were used. The whole amplification, library preparation and sequencing workflow including prior quality control was performed by Novogene Inc (Beijing, China). Briefly, quality control (QC) was performed on a 1% agarose gel electrophoresis. Tick DNA was subsequently diluted to 1 ng/µL using sterile water and subjected to PCR using Phusion High-Fidelity PCR Master Mix (New England Biolabs), followed by agarose gel electrophoresis. Only samples showing a bright band between 400 and 460 bp were used for library generation. The purification of PCR product mixtures was performed using the Qiagen Gel Extraction kit (Qiagen, Germany). Libraries were generated by NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs) and quantified by Qubit and quantitative PCR (qPCR). Sequencing was performed on a Novaseq 6000 (Illumina) with a sequencing depth of 50 k raw reads per sample.

2.5. Bioinformatics and statistical analyses of NGS data

Processing of the sequence data including Operational Taxonomic Unit clustering was performed by Novogene Inc. Paired-end reads were merged by FLASH software (V1.2.7.) (Magoc and Salzberg, 2011) and further quality-filtered by QIIME (V1.7.0.) (Caporaso et al., 2010). Chimera sequences were identified and eliminated using the UCHIME algorithm (Edgar et al., 2011). Sequence analysis was performed by Uparse software (V7.0.1001). Mothur software was used against SSUrRNA database of the SILVA reference database (Wang et al., 2007; Quast et al., 2013) for species annotation, with a cut-off at ≥97% similarity. MUSCLE (3.8.31) (Edgar, 2004) software was used for further phylogenetic analyses. Alpha and beta diversity were analyzed by R (V 4.2.2). For data processing, the phyloseq package was used (McMurdie and Holmes, 2013). We further used the decontam package to identify possible contaminants by comparing the OTU abundance of negative controls to samples (Davis et al., 2018). Here, the prevalence method with a threshold of 0.5 was used. This was followed by trimming OTUs which were not present in any samples from our data subset. Further analysis was performed with this data subset. Alpha diversity for species richness included Chao1 and abundance-based coverage estimator (ACE), while for species diversity Shannon and Simpson index, the index of sequencing depth and observed species were included. Here, the Wilcoxon test was performed for statistical analysis and the graphs were computed by phyloseq package, ggplot2 and ggpubr packages (Wickham, 2011; McMurdie and Holmes, 2013; Kassambara, 2020). Beta diversity measures included weighted and unweighted UniFrac, focusing on relative abundances by using the Bray-Curtis distance measure computed by the phyloseq package. Further, the Non-Metric Multidimensional Scaling (NMDS) was computed by phyloseq package and a Principal Component Analysis (PCA) was computed and visualized by the MicrobiotaProcess package (McMurdie and Holmes, 2013; Xu and Yu, 2022). Statistical analyses for dissimilarity measures were performed by Permutational Multivariate Analysis of Variance (PERMANOVA) by the vegan package ($n = 999$ permutations; Oksanen et al., 2013). For beta diversity graphs, the phyloseq package was used (McMurdie and Holmes, 2013). For all statistical tests, a statistical significance level at $p < 0.05$ was set.

TABLE 1 Primers used in this study.

Organism	Target gene	Primer name	Sequence (5′–3′)	Annealing temperature for PCR (°C)	Product size (bp)	Reference
Ca. Midichloria mitochondrii	*gyrB*	gyrB-F	CTTGAGAGCAGAACCACCTA	61.5	125	Sassera et al. (2008)
		gyrB-R	CAAGCTCTGCCGAAATATCTT			
Ixodes ricinus	*Calreticulin*	calF	ATCTCCAATTTCGGTCCGGT	64.5	109	
		calR	TGAAAGTTCCCTGCTCGCTT			
Rickettsia spp.	*gltA*	Rickettsia_gltA_F1	GCTCTTCTCATCCTATGGCTATTA	59.1	499	This study
		Rickettsia_gltA_R2	TCCTTAGCTTTAGCTATATATTTAGG			
Rickettsia helvetica	*gltA*	Rhelvetica_qPCR_F2	GGAAGCAGACTACAAACTTACTGC	–	173	This study
		Rhelvetica_qPCR_R2	CTTTATATTTCGTACAAGGCGTTG			
Spiroplasma spp.	*rpoB*	Spiro_rpoB_qPCR_F1	CCAAAAGGTCAAACACAATCAAC	62.1	127	This study
		Spiro_rpoB_qPCR_R1	TACCTTGAACAATTCCAGCACC			
Spiroplasma	*gyrA*	Spixo_gyrA_F2	CCAGATGCAAGAGATGGATTG	56	561	Binetruy et al. (2019a)

2.6. Sequencing

The *Rickettsia* and *Spiroplasma* spp. detected by bacterial 16S rRNA sequencing were further identified by amplifying a ~ 499 bp region of the *Rickettsia gltA* gene and a ~ 561 bp fragment of the *Spiroplasma* DNA gyrase subunit A (*gyrA*) gene (Table 1), followed by amplicon sequencing (LGC Genomics, Berlin, Germany) and BLASTn analysis.

2.7. Plasmid DNA for standard curves

To generate plasmid DNA for standard curves that were used in the qPCR, PCR products were amplified using S7 Fusion polymerase (Mobidiag, Espoo, Finland). Each reaction mixture consisted of 5 µL of 5X HF buffer, 1 µL of each forward and reverse primer (10 µM), 0.5 µL of dNTP (2 mM), 0.25 µL polymerase, 1 µL DNA and nuclease-free water up to a reaction volume of 25 µL. Cycling conditions were 98°C for 8 s followed by 35 cycles of 94°C for 5 s, annealing for 20 s, and 72°C for 15 s, with a final extension step at 72°C for 1 min. Amplicons of the expected size were cleaned using the DNA Clean & Concentrator-5 kit (Zymo Research, Freiburg, Germany) and cloned in the pSC-B-amp/kan vector (Strataclone Blunt Cloning Kit, Agilent). Plasmids were isolated using the GenUp Plasmid Kit (Biotechrabbit, Berlin, Germany) and sequenced by LGC Genomics. Ten-fold serial dilution stocks with known copies of each plasmid DNA were prepared and stored at −20°C.

2.8. Quantification of endosymbionts

To measure the relative level of three of the most abundant bacteria identified by bacterial 16S rRNA sequencing in all unfed life stages (including the same female tick DNA samples as used for 16S sRNA sequencing), we performed qPCRs using the primers listed in Table 1. Novel primers were manually designed using NetPrimer software[1] based on nucleotide sequence alignments made in BioEdit 7.0.5.3.[2] qPCR reaction mixtures consisted of 10 µL Luna Universal mastermix (New England Biolabs, Frankfurt am Main, Germany), 1 µL of each primer (10 µM), 1 µL DNA and 7 µL nuclease-free water. Cycling conditions were 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and annealing/elongation at 60°C for 1 min in a CFX96 cycler (Bio-Rad Laboratories GmbH, Feldkirchen, Germany). All samples were run in technical duplicates. A no-template control and serial dilutions of plasmid DNA were included in each run. Results were normalized against the *I. ricinus calreticulin* (*cal*) gene as a reference gene in CFX Maestro software (Bio-Rad).

2.9. Statistical analyses for feeding and quantitative PCR data

For tick feeding experiments, analyses were computed in R (V 4.2.2.) (R Core Team, 2013) either by the Mann–Whitney U test or the *t*-test with Welch-Correction depending on normal distribution, the Z-test for proportions followed by degrees of freedom (df) and Chi-Square ($\chi 2$). Confidence intervals (CI) were computed at the 95% level and CIs for proportions were computed by the binom. wilson function from the epitools package (V 0.5–10.1). Graphs of feeding parameters and relative bacterial abundance were produced using the ggplot2 package (3.3.1) with a significance level of $p < 0.05$ (Wickham, 2011). The graphs and data analysis concerning the qPCR data on target gene/ housekeeping gene ratio were performed

1 http://www.premierbiosoft.com/netprimer/

2 http:///www.mbio.ncsu.edu/BioEdit/bioedit.html

in GraphPad Prism 9.3.1 (Graphpad Software Inc., La Jolla, United States), for which a Mann–Whitney test was performed and a value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Artificial tick feeding with and without antibiotics and tick feeding on cattle

All consecutive life stages (larvae, nymphs, adults) were successfully fed in the IVG$^+$ and the IVG$^-$ group. For the C group, feeding of consecutive larvae and nymphs on calves was also successful but feeding of the adult ticks on a calf failed for unknown reasons. Adults originating from a different larval batch to obtain comparative data for the *in vitro* feeding were used instead. Since only the microbiome of unfed larvae, unfed nymphs and unfed adult ticks originating from the same larval batches were analyzed, this did not affect the results from 16S rRNA sequencing or the qPCR analysis.

Overall, we fed an estimated number of 1,505 (IVG$^-$), 709 (IVG$^+$), and 1160 (C) F_0-larvae. For the consecutive feeding of nymphs that molted from these larvae, a total of 96 (IVG$^-$), 136 (IVG$^+$) and 200 (C) nymphs were used. These ticks were again fed after molting as adults. Here, 25 (IVG$^-$), 10 (IVG$^+$) and 100 (C) females were fed.

3.1.1. Larvae feeding

In general, we observed a statistically significant positive effect of gentamicin supplementation in the IVG$^+$ larvae group compared to IVG$^-$ for the proportion of engorged larvae and molting proportion per engorged larvae (Figure 1A; Supplementary Table S1). Although IVG$^+$ larvae showed a statistically significant higher engorgement proportion than control ticks, the molting proportion was significantly higher for larvae fed on calves.

3.1.2. Nymphal feeding

The positive effect of gentamicin observed for *in vitro* fed larvae was not seen for *in vitro* fed nymphs. Here, nymphs of the IVG$^-$ group had a higher engorgement- and molting proportion (Figure 1B; Supplementary Table S2). The weight of engorged nymphs and unfed females did not significantly differ between IVG$^+$ and IVG$^-$ (Figures 2A,B; Supplementary Table S2). No significant difference was observed for the engorgement proportion between IVG$^+$ nymphs compared to C group nymphs, but nymphs of the IVG$^+$ group did have lower engorgement weights, molting proportion and unfed female weights.

3.1.3. Adult feeding

IVG$^+$ females did not significantly differ in terms of detachment proportion and detachment weight compared to C group females. However, egg masses and the proportion of viable larvae-producing females were higher for the C group when compared to IVG$^+$. Parameters of females did not significantly differ between the IVG$^+$ and IVG$^-$ (Figures 2C,D; Supplementary Table S3).

3.2. Bacterial 16S rRNA sequencing

The DNA concentration from 14/19 unfed female tick samples (IVG$^+$: 6/6, IVG$^-$: 1/5, C: 7/8) was high enough to pass quality control analysis and was subjected to 16S rRNA amplification and sequencing. The DNA concentration of some extracts from individual ticks, in particular in the IVG$^-$ female group without gentamicin, was considered to be too low for 16S rRNA sequencing. DNA extracted from pools of larvae, individual nymphs and males did not pass the quality control of the service provider and were not sequenced. Due to the low sample size, IVG$^-$ was excluded for further analysis.

In total, 986,684 reads, resulting in 984,005 effective tags after quality filtering and comparison with reference databases were obtained (Supplementary material S1). The mean number of reads per sample in unfed females from the IVG$^+$ and C groups were 76,004 (± 11,109) and 74,064 (± 4,493), respectively. Removing contaminants by decontam package resulted in a mean library size of 75,968 (± 11,109) for IVG$^+$ and 73,820 (± 4,439) for C group. Overall, females fed as larvae and nymphs on calves had a more diverse microbiome composition compared to females of the IVG$^+$ group (Figure 3). All samples except the single IVG$^-$ female, contained *Rickettsia* and *M. mitochondrii* OTUs. *Streptomyces* ($n = 13/14$) and *Spiroplasma* ($n = 12/14$) were also detected in most samples.

The relative abundance of the bacteria differed between the two groups of females. As shown in Figure 3, the relative abundance of IVG$^+$ reared female ticks was dominated by *Rickettsia* OTUs, while females reared on calves had a higher relative abundance of *M. mitochondrii* and *Spiroplasma*.

The diversity indices were statistically compared between IVG$^+$ and C females (Supplementary Figure S1). In general, there was a tendency for higher diversity means within the C female group, which were significant for the Shannon (Wilcoxon, $p = 0.008$) and the Simpson index (Wilcoxon, $p = 0.004$). Furthermore, PCoA of unweighted and weighted UniFrac and NMDS distances of IVG$^+$ females showed a narrow spectrum in comparison to the much broader cluster of control females (Supplementary Figure S2). This bacterial community structure differed between IVG$^+$ and C females (weighted UniFrac: df = 1(11), $p = 0.002$, unweighted UniFrac: df = 1 (11), $p = 0.53$).

3.3. Sequencing of *Rickettsia* and *Spiroplasma* spp.

The *Rickettsia* species detected by 16S rRNA sequencing was identified as *R. helvetica* following sequencing of the *gltA* gene. The *gltA* sequence was 100% identical (499/499 nt) to that of the *R. helvetica* C9P9 reference strain (GenBank Accession Number

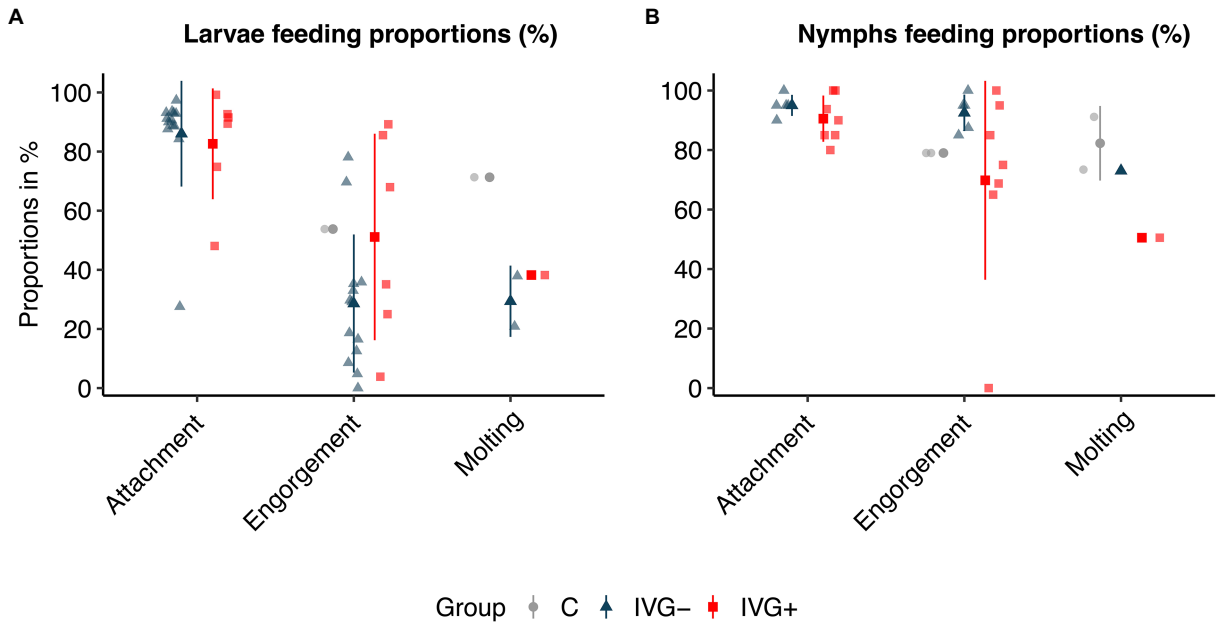

FIGURE 1
Attachment, engorgement, and molting proportions of *Ixodes ricinus* larvae **(A)** and nymphs **(B)**. Proportions are presented in % per feeding unit or per animal experiment. Bars present means ± standard deviation. IVG$^+$, *in vitro* feeding with gentamicin; IVG$^-$, *in vitro* feeding without gentamicin; C, control feeding *in vivo* on calves.

CM001467). The partial *gyrA* sequence of the *Spiroplasma* species showed most identity (506/507 nt, 99.8%) to *Spiroplasma ixodetis* isolated from *I. ricinus* (MK267048) and *Ixodes uriae* (MK267049).

3.4. Quantitative analyses of bacterial loads

The qPCR data processing was limited to the three main bacterial species detected by 16S rRNA sequencing: *R. helvetica*, *M. mitochondrii*, and *Spiroplasma* species.

The qPCR data for the three F_0-larvae batches showed no significant differences for the main bacterial species: all three batches were positive for *M. mitochondrii* and *R. helvetica*, but negative for *Spiroplasma* (Supplementary Table S4).

The *M. mitochondrii* abundance was significantly higher in unfed IVG$^+$ and C females compared to the corresponding nymphal stages (Mann–Whitney test, $p = 0.0034$ and $p = 0.0129$, respectively), but this was not the case for unfed IVG$^-$ females and nymphs (Mann–Whitney test, $p = 1.0$). *Midichloria mitochondrii* was not detected in male ticks. The *M. mitochondrii* bacterial loads were significantly higher for the C females compared to IVG$^-$ (Mann–Whitney test, $p = 0.0132$; Figure 4).

Rickettsia helvetica was also present in all F_0-larvae groups (Figure 5). The relative number of *R. helvetica* bacteria increased in the nymphal IVG$^+$ and IVG$^-$ ticks, but not in the C group. The relative number of *R. helvetica* was significantly higher in IVG$^+$ nymphs compared to the C group (Mann–Whitney test, $p = 0.0043$), but did not statistically differ compared to IVG$^-$ (Mann–Whitney test, $p = 0.30$), although some IVG$^-$ nymphs tested negative for *R. helvetica* by qPCR. IVG$^+$ and IVG$^-$ ticks remained positive as adults at similar levels as nymphs, with significantly higher levels in the IVG$^+$ group compared to the C and IVG$^-$ group as both females (Mann–Whitney test, $p = 0.0298$ and $p = 0.0398$, respectively) and males (Mann–Whitney test, $p = 0.0225$ and $p = 0.0225$, respectively).

Although *Spiroplasma* was detected by 16S rRNA sequencing in most (5/6) IVG$^+$ females (albeit in low numbers) and in all C females in higher numbers, they were only detectable by qPCR in the C females (Figure 6).

4. Discussion

4.1. Effects on tick feeding

In general, ticks from IVG$^+$ and IVG$^-$ showed lower engorgement and detachment weights and a longer feeding duration compared to ticks from the C group. This was in accordance to previous work, where artificially fed ticks were compared to ticks fed on calves (Militzer et al., 2021). The highest proportion of larvae that engorged was found in the IVG$^+$ group. It should be noted that although all life stages of *I. ricinus* can feed on large ruminants, they are not commonly used as experimental animals for the feeding of *I. ricinus* larvae, so there is little information available on the feeding efficacy of larvae on cattle (Jaenson et al., 1994; Hofmeester et al., 2016; Levin and Schumacher, 2016). This may explain the limited engorgement proportion observed for the control group larvae. We nonetheless

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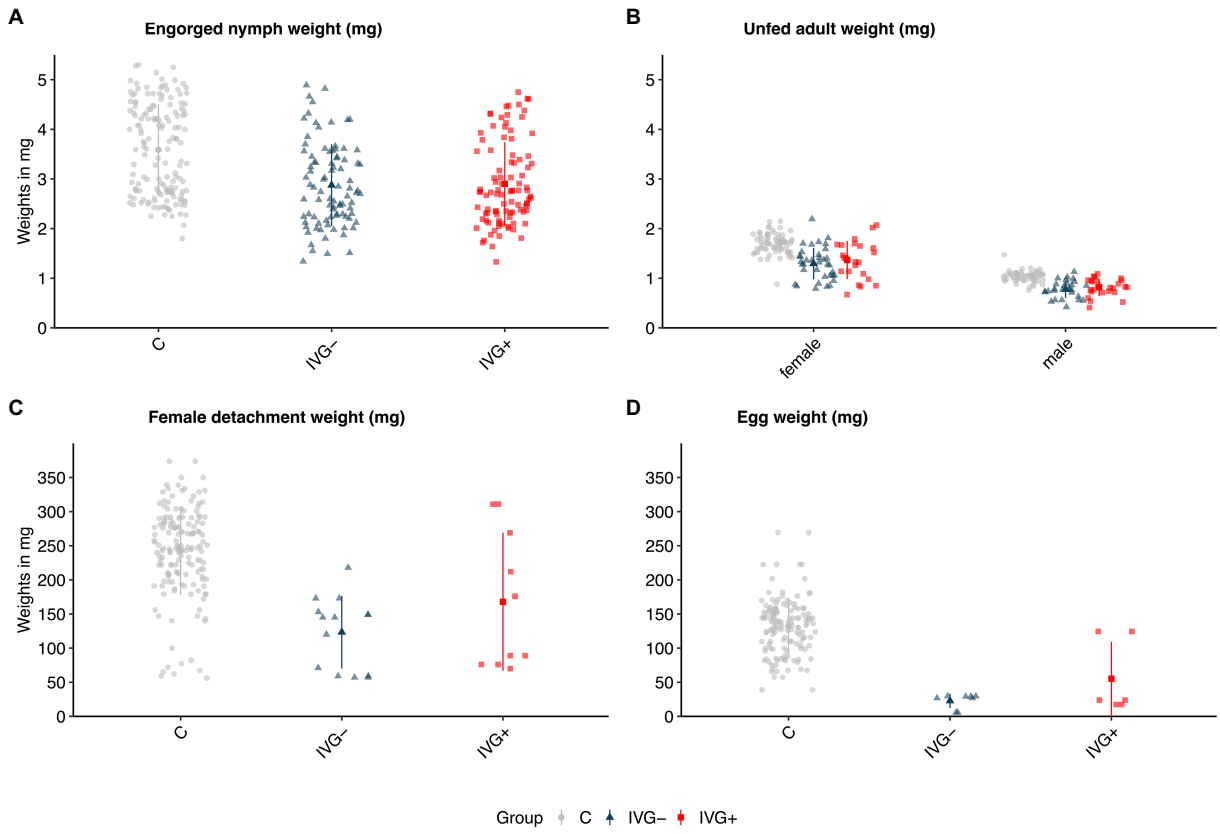

FIGURE 2
Weights (in mg) of **(A)** engorged *Ixodes ricinus* nymphs, **(B)** molted unfed adult *I. ricinus* females and males, **(C)** engorged and detached *I. ricinus* females, and **(D)** egg batches of *I. ricinus* females. Bars present means ± standard deviation. IVG$^+$, *in vitro* feeding with gentamicin; IVG$^-$; *in vitro* feeding without gentamicin; C, control feeding *in vivo* on calves.

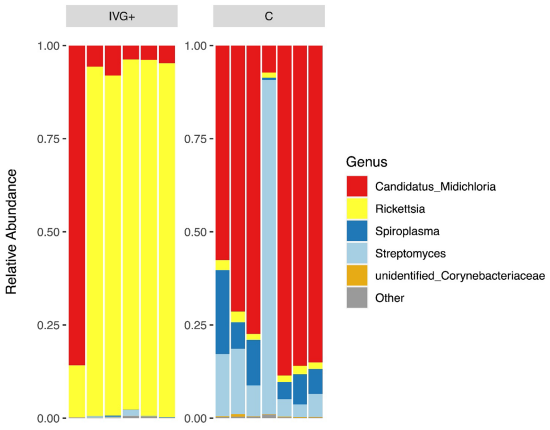

FIGURE 3
Relative abundances of the top five genera for *in vitro* reared females with gentamicin supplementation (IVG$^+$, n=6), and *in vivo* reared females (C, n=7).

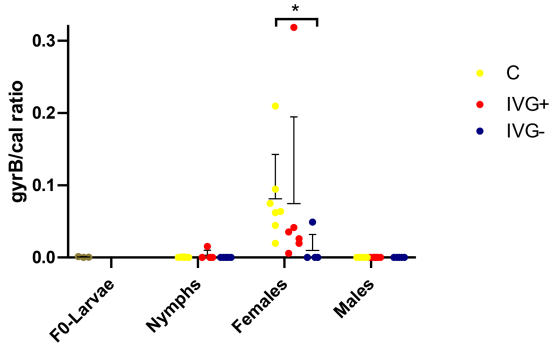

FIGURE 4
qPCR profiles (relative expression) of *Midichloria mitochondrii*. Each dot represents the relative *gyrB* expression in a single sample. The tick *calreticulin* gene was used as a housekeeping gene. Bars represent mean with the standard deviation shown in error bars. Statistical analysis by Mann–Whitney test performed in Graphpad. IVG$^+$, *in vitro* feeding with gentamicin; IVG$^-$, *in vitro* feeding without gentamicin; C, control feeding *in vivo* on calves. *p<0.05.

preferred to use only cattle as hosts or blood source for all experiments to reduce variation between experiments, as the source of the blood meal was previously shown to affect tick feeding and molting (Koch and Hair, 1975; Brunner et al., 2011). The molting proportions were lower for IVG$^+$ and IVG$^-$ fed larvae compared to the C larvae, which might be explained by their

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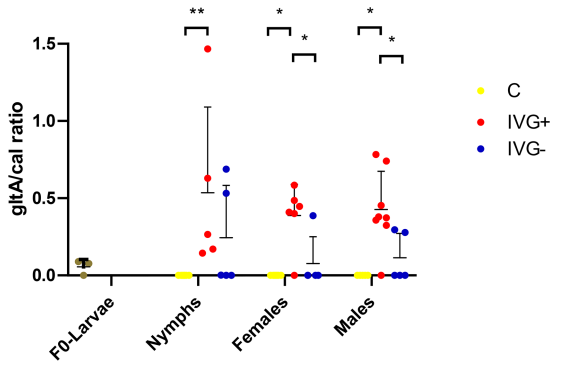

FIGURE 5
qPCR profiles (relative expression) of *Rickettsia helvetica*. Each dot represents the relative *gltA* expression in a single sample. The tick *calreticulin* gene was used as a housekeeping gene. Bars represent mean with the standard deviation shown in error bars. Statistical analysis by Mann–Whitney test performed in Graphpad. IVG$^+$, *in vitro* feeding with gentamicin; IVG$^-$, *in vitro* feeding without gentamicin; C, control feeding *in vivo* on calves. *p<0.05, **p<0.01.

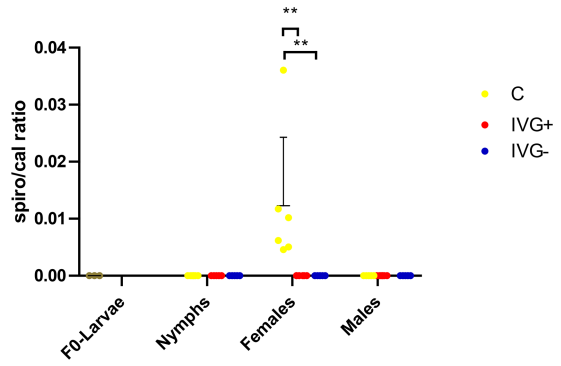

FIGURE 6
qPCR profiles (relative expression) of *Spiroplasma* spp. Each dot represents the relative *rpoB* expression in a single sample. The tick *calreticulin* gene was used as a housekeeping gene. Bars represent mean with the standard deviation shown in error bars. Statistical analysis by Mann–Whitney test performed in Graphpad. IVG$^+$, *in vitro* feeding with gentamicin; IVG$^-$, *in vitro* feeding without gentamicin; C, control feeding *in vivo* on calves. **p<0.01.

significantly lower engorgement weight. Previous studies on *Amblyomma americanum* showed that the molting success of larvae and nymphs was associated to their engorgement weight; ticks that failed to reach a "critical" engorgement weight did not molt (Koch, 1986). Similar results were observed for *Rhipicephalus sanguineus* nymphs (Ben-Yosef et al., 2020).

The supplementation of the blood meal with gentamicin and B vitamins resulted in higher engorgement or detachment weights, in particular for females (IVG$^+$). In general, female ticks typically have a longer feeding duration than juvenile ticks, which increases the risk of bacterial contamination of the blood meal. This may have led to a reduction in the blood meal quality and prevented the ticks from fully engorging. Juvenile *I. ricinus* ticks did feed successfully without antibiotics, corroborating results of other studies where antibiotics were also omitted from the blood meal, for instance to prevent possible interference of antibiotics with pathogen acquisition or transmission (e.g., Waladde et al., 1993; Koci et al., 2018; Korner et al., 2020). It is important to note here that data on the artificial feeding of consecutive hard tick life stages is very limited (Kuhnert et al., 1995; Militzer et al., 2021). A possible cumulative effect of antibiotics on ticks and their endosymbionts cannot be ruled out and need to be studied further.

4.2. Effects on the microbiome diversity

In our study, the microbiome of female ticks fed as larvae and nymphs on the ears of calves was more diverse than that of the IVG$^+$ group and possibly from that of the IVG$^-$ group, for which only limited 16S rRNA sequencing data was available. This difference in variety could be explained by the feeding process: C ticks fed on calves were exposed to a wider variety of bacteria, for instance from the microbiome of the bovine skin and cerumen, compared to the IVG$^+$ and IVG$^-$ groups that were fed in a relatively sterile laboratory environment. They may therefore have acquired a more diverse set of bacteria from their environment by oral or cuticular routes compared to the IVG$^+$ ticks. In addition, the microbiome variety could have been reduced by the exposure of IVG$^+$ ticks to gentamicin, but the lack of sufficient data for the IVG$^-$ group prevents the drawing of definitive conclusions in this regard.

Interestingly, most of the IVG$^+$ and IVG$^-$ females (n = 6/7) had a high relative abundance of *R. helvetica* and low relative abundances of *M. mitochondrii* and *Spiroplasma* spp. This was in contrast to the C females, where most ticks (n = 6/7) showed a high relative abundance of *M. mitochondrii*, a finding supported by qPCR data. However, when C and IVG$^+$ groups were directly compared, the difference was not significant. This was mostly due to one sample of the IVG$^+$ group, which had an exceptionally high *M. mitochondrii/calreticulin* ratio of 0.318, corroborating its 16S rRNA sequencing result (Figures 3, 4).

Midichloria mitochondrii is abundant in various tick species collected from the field, with a reported prevalence ranging from 54.8 to 100% in *I. ricinus* females (Lo et al., 2006; Sassera et al., 2006; Duron et al., 2017; Garcia-Vozmediano et al., 2021). A prevalence below 100% may suggest that the symbiosis is not obligatory for *I. ricinus* to survive, or that other symbionts take over this role when *Midichloria* is absent (Krawczyk et al., 2022a). The number of *Midichloria* was shown to decrease after the molt of *I. ricinus* and increase during blood meal intake, suggesting that it may be relevant for tick development, for instance by providing the tick with essential nutrients that are missing in the blood meal (Sassera et al., 2008; Olivieri et al., 2019). In females, *Midichloria* have mainly been found in ovaries, which ensures its maternal transition (Epis et al., 2013; Olivieri et al., 2019). In our study, the analysis for *M. mitochondrii* was performed in unfed ticks, i.e., prior to feeding, which may explain the relatively low bacterial loads found compared to previous reports (Sassera et al., 2008).

Of note, the laboratory tick colony originated from ticks flagged in a study site that was previously shown to have a low *Midichloria* prevalence (36.4%) in nymphs (Garcia-Vozmediano et al., 2021). Furthermore, *M. mitochondrii* was not detected in male ticks, corroborating previous reports describing a low *Midichloria* abundance in males (Lo et al., 2006; Sassera et al., 2008; Lejal et al., 2020; Garcia-Vozmediano et al., 2021). Future male nymphs have also been reported to have lower *Midichloria* loads than future female-nymphs (Epis et al., 2013; Daveu et al., 2021). Since differentiation between future male and future female was not possible at the time of testing, this may also have influenced the detection of *M. mitochondrii* in nymphs in our study.

Ixodes spp. ticks have been shown to harbor several *Rickettsia* species (Kurtti et al., 2015; Hajduskova et al., 2016; Duron et al., 2017; Nováková and Šmajs, 2018). So far, *R. helvetica*, *Candidatus* R. mendelii, *R. monacensis*, *R. raoultii*, *R. slovaca*, and *Candidatus* R. thierseensis have been detected in *I. ricinus* ticks, with *R. helvetica* being the most common *Rickettsia* species found (Simser et al., 2002; Hajduskova et al., 2016; Schötta et al., 2017, 2020). The 16S rRNA sequencing results and additional *gltA* sequencing suggest that the *I. ricinus* ticks used for this study only contained *R. helvetica*. As the sequence of the 16S rRNA V3-V4 region of *R. helvetica* differs from that of the other *Rickettsia* species that have been associated with *I. ricinus*, the presence of these *Rickettsia* spp. in ticks used for this study is not plausible.

Rickettsia spp. are gram-negative intracellular alpha-proteobacteria that can be categorized in several groups (Salje, 2021). *Rickettsia helvetica* belongs to the spotted fever group (SFG), a group that contains several pathogenic species such as *R. rickettsi* and *R. conorii*, the causal agents of Rocky Mountain spotted fever and Mediterranean spotted fever, respectively, but also contains *Rickettsia* species of undetermined pathogenicity. Even though previous literature reported the detection of *R. helvetica* in a small number of diseased humans, disease causation has not been convincingly demonstrated and the pathogenicity of *R. helvetica* remains to be determined (Nilsson et al., 1999, 2010; Azagi et al., 2020).

qPCR results showed that *R. helvetica* was the predominant bacterial species in IVG$^-$ and IVG$^+$ groups, confirming the 16S rRNA sequencing data for IVG$^+$ (Figures 3, 5). This was a striking finding, as all three F_0-larvae batches with which the study started contained similar amounts of *R. helvetica* (Figure 5 and Supplementary Table S4). This suggests that artificial feeding led to a positive selection for *R. helvetica* in the majority of the analyzed samples. We hypothesize that this may have been caused by interactions between *R. helvetica* and the microbiome, which was less varied compared to the C group, leading to a dysbiosis that could have facilitated *R. helvetica* colonization of the ticks. Interestingly, a recent study described a significant reduction in the microbiota diversity in *I. ricinus* nymphs collected from humans that were infected with *R. helvetica* (Maitre et al., 2022). The authors hypothesized that *R. helvetica* may modulate the tick microbiome to facilitate colonization whereas our results raise the question if a high *R. helvetica* abundance could not actually be the result of a reduced tick microbiome diversity. The presence of *R. helvetica* in bovine blood used as a blood meal source could be an alternative explanation for the increased *R. helvetica* abundance. Although there are no reports on the detection of *R. helvetica* in bovine blood, it has been detected in the blood of other ruminants such as domestic goats (*Capra hircus*), roe deer (*Capreolus capreolus*) and sika deer (*Cervus nippon yeiensis*; Inokuma et al., 2008; Stefanidesova et al., 2008; Rymaszewska, 2018). However, the original blood samples used for the artificial feeding were not available anymore to test this hypothesis. Although blood collected from the same donor cattle several months after the use of their blood for artificial feeding of larvae tested negative for the presence of *R. helvetica* DNA by PCR (results not shown), this alternative hypothesis cannot be fully excluded. The observed high abundance of *R. helvetica* in IVG$^+$ and IVG$^-$ ticks could be useful for experimental studies in which a high pathogen abundance in ticks is advantageous. On the other hand, it also shows that the composition of the tick microbiome should be taken into account in ATFS acquisition and transmission studies, as ATFS itself may have a direct effect on the tick microbiome and tick-borne pathogen abundance. Successful colonization of *Ixodes* ticks with the causal agent of Lyme Borreliosis, *Borrelia burgdorferi sensu stricto*, has for instance been associated with a higher microbiome diversity (Narasimhan et al., 2017; Sperling et al., 2020). This should be considered in the experimental design of ATFS acquisition and transmission models for this pathogen.

A third species that was particularly abundant in the C females was *Spiroplasma* (Figure 6). *Spiroplasma ixodetis* is considered to be a facultative symbiont and has previously been detected in *I. ricinus* ticks, but its effect on ticks has not been clarified yet (Duron et al., 2017; Lejal et al., 2021). *Spiroplasma* spp. in *Ixodes* ticks are thought to maternally inherited (Beliavskaia et al., 2021) and although we did detect *Spiroplasma* OTUs in both the IVG$^+$ and C females, we could not detect *Spiroplasma* DNA by qPCR in the F_0-larvae to confirm transovarial transmission. However, this may also have been caused by limitations in the sensitivity of the used qPCR for the detection of *Spiroplasma*. A previous study on the microbiome of *I. ricinus* nymphs collected from the vegetation near Paris, France, showed a decreased abundance of *Spiroplasma* in *Rickettsia*-positive samples (Lejal et al., 2021), which corroborates with our findings where *Spiroplasma* was not detected by qPCR in ticks with a high *R. helvetica* abundance. This negative association is suggestive of competition or niche partitioning between *Spiroplasma* and *R. helvetica* (Krawczyk et al., 2022a,b).

Endosymbionts such as *Midichloria* are thought to play an important role in tick biology by providing essential B vitamins to ticks (Duron et al., 2018; Duron and Gottlieb, 2020). The most common bacteria associated with providing essential B vitamins other than *Midichloria* are *Coxiella*-like endosymbionts, *Francisella*, and some *Rickettsia* spp (Hunter et al., 2015; Duron et al., 2017). Although the production of a core set of B vitamins (biotin, riboflavin and folate) is usually associated with a single nutritional symbiont for each tick species (Duron et al., 2017), it was recently suggested that in some tick species a dual

endosymbiosis occurs whereby a second endosymbiont provides B vitamin components that the other endosymbiont cannot produce (Buysse et al., 2021). Previous analyses showed that the genome of *M. mitochondrii* contains genes for the synthesis of biotin and folate, but does not seem to have all genes required for the synthesis of riboflavin (Buysse et al., 2021). It leaves the question from which source *I. ricinus* obtains riboflavin, provided that the levels found in blood are insufficient. The genome of *R. helvetica* does not have a functional riboflavin pathway and it would be interesting to examine if the genomes of other bacteria associated with *I. ricinus*, such as *S. ixodetis*, *Rickettsiella* or perhaps *Streptomyces* species would have functional B vitamin synthetic pathways. If so, this might also explain how *I. ricinus* ticks in which *M. mitochondrii* is absent obtain essential B vitamin components. We also observed a negative association between *Midichloria* and *R. helvetica*. The same negative association was found in a previous study in which nearly 14,000 questing *I. ricinus* nymphs were screened by qPCR for tick-associated microorganisms (Krawczyk et al., 2022b). In contrast, other studies reported a positive association between *Midichloria* and *Rickettsia* spp., both in questing ticks and ticks collected from humans (Budachetri et al., 2018; Lejal et al., 2021; Maitre et al., 2022). These contrasting results may in part be explained by factors found to be of influence the microbiome composition of ticks that differed between the studies, such as environmental temperature and the identity of hosts on which the ticks fed (Swei and Kwan, 2017; Thapa et al., 2019).

A major limitation of this study is the low sample size for the 16S rRNA sequencing, due to low DNA yields. It has previously been reported that the extraction of DNA from single *I. scapularis* ticks and samples with a low biomass may result in low yields (Ammazzalorso et al., 2015). To overcome the lack of 16S rRNA sequencing data for the juvenile life stages, additional qPCRs were performed for larvae and nymphs, in which constant results for the tick *calreticulin* gene were obtained (Supplementary Table S4). It is known that low biomass samples are at a higher risk for contamination sequences than higher biomass samples (Salter et al., 2014; Eisenhofer et al., 2019; Lejal et al., 2020). Pooling of ticks would have been an alternative to increase DNA yields for sequencing and to have robust samples against biases and contamination challenges. Although pooling of ticks gives only limited insights in microbial communities and diversities, it could have been an alternative in combination with qPCR (Krawczyk et al., 2022a). Future studies should take these observations into account.

Another limitation of this study is the relatively low number of ticks that could consecutively be fed from the larval to the adult stage. The resulting sample size was too low to conduct further statistical analyses on eggs and F_1-larvae. The absence of B vitamin components in the blood meals offered to the *in vitro* fed larvae and nymphs could have negatively influenced tick fitness and development at these stages. The optimal dose of B vitamin supplementation and its effect on the larvae and nymphs should be examined in more detail in future studies aimed at optimizing the artificial feeding of *I. ricinus*.

In conclusion, we examined the microbiome of *I. ricinus* under different experimental conditions by feeding all consecutive life stages of *I. ricinus* by ATFS on blood meals with (IVG^+) or without gentamicin (IVG^-) and comparing the feeding parameters to those of ticks fed simultaneously on calves (C). The tick microbiome composition was studied by 16S rRNA sequencing and qPCRs for *M. mitochondrii*, *R. helvetica*, and *Spiroplasma* spp. The results showed a shift of the ticks' microbiome, with the symbiont *M. mitochondrii* being the dominant genus for females fed as larvae and nymphs on calves and *R. helvetica* being the most abundant bacteria in females that were fed as juveniles *in vitro*. IVG^- females showed significant lower loads of *M. mitochondrii* compared to the other groups. *Spiroplasma* spp. loads also differed: while exclusively detected in C female ticks by qPCR, 16S rRNA sequencing results also showed low relative abundances in IVG^+ females. Collectively, the results showed that the employed feeding techniques affect the fecundity and microbiome composition of ticks, with a decreased microbiome diversity in artificially fed ticks fed on blood supplemented with gentamicin. These effects should be taken into account in studies employing ATFS.

Data availability statement

The data presented in the study are deposited in the NCBI BioProject repository, accession number PRJNA905798.

Ethics statement

All animal experiments were approved by the regional authorities for animal experiments (LaGeSo, Berlin, 0387/17).

Author contributions

AN, NM, and SPS conceptualized this study. NM carried out methodology. AN and NM performed the formal analysis and wrote the original draft. All authors read and approved the final manuscript.

Funding

This study was funded by the German Federal Ministry of Education and Research (BMBF, grant number 01KI1720) as part of the Junior Research Group "Tick-borne Zoonoses." This research was further funded by Deutsche Forschungsgemeinschaft (German Research Foundation, DFG) through the Research Training Group GRK 2046 "Parasite Infections: From experimental models to natural systems" (NM and SPS associated Ph.D. candidates/AN Senior Researcher).

Acknowledgments

We would like to thank Peggy Hoffmann-Köhler, Anne-Kathrin Hübner, Khawla Elati, David Omondi, and Samira Schlesinger for excellent technical assistance, Andrea Feßler for microbiological support, and Alexander Bartel for statistical support. We acknowledge support by the Open Access Publication Initiative of Freie Universität Berlin.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1050063/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 11 January 2023
DOI 10.3389/fmicb.2022.1113237

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

RECEIVED 01 December 2022
ACCEPTED 21 December 2022
PUBLISHED 11 January 2023

CITATION
Goffredi SK, Appy RG, Hildreth R and deRogatis J (2023) Marine vampires: Persistent, internal associations between bacteria and blood-feeding marine annelids and crustaceans. *Front. Microbiol.* 13:1113237. doi: 10.3389/fmicb.2022.1113237

Marine vampires: Persistent, internal associations between bacteria and blood-feeding marine annelids and crustaceans

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Persistent bacterial presence is believed to play an important role in host adaptation to specific niches that would otherwise be unavailable, including the exclusive consumption of blood by invertebrate parasites. Nearly all blood-feeding animals examined so far host internal bacterial symbionts that aid in some essential aspect of their nutrition. Obligate blood-feeding (OBF) invertebrates exist in the oceans, yet symbiotic associations between them and beneficial bacteria have not yet been explored. This study describes the microbiome of 6 phylogenetically-diverse species of marine obligate blood-feeders, including leeches (both fish and elasmobranch specialists; e.g., *Pterobdella, Ostreobdella,* and *Branchellion*), isopods (e.g., *Elthusa* and *Nerocila*), and a copepod (e.g., *Lernanthropus*). Amplicon sequencing analysis revealed the blood-feeding invertebrate microbiomes to be low in diversity, compared to host fish skin surfaces, seawater, and non-blood-feeding relatives, and dominated by only a few bacterial genera, including *Vibrio* (100% prevalence and comprising 39%–81% of the average total recovered 16S rRNA gene sequences per OBF taxa). *Vibrio* cells were localized to the digestive lumen in and among the blood meal for all taxa examined *via* fluorescence microscopy. For *Elthusa* and *Branchellion, Vibrio* cells also appeared intracellularly within possible hemocytes, suggesting an interaction with the immune system. Additionally, *Vibrio* cultivated from four of the obligate blood-feeding marine taxa matched the dominant amplicons recovered, and all but one was able to effectively lyse vertebrate blood cells. Bacteria from 2 additional phyla and 3 families were also regularly recovered, albeit in much lower abundances, including members of the Oceanospirillaceae, Flavobacteriacea, Porticoccaceae, and unidentified members of the gamma- and betaproteobacteria, depending on the invertebrate host. For the leech *Pterobdella*, the Oceanospirillaceae were also detected in the esophageal diverticula. For two crustacean taxa, *Elthusa* and *Lernanthropus*, the microbial communities associated with brooded eggs were very similar to the adults, indicating possible direct transmission. Virtually nothing is known about the influence of internal bacteria on the success of marine blood-feeders, but this evidence suggests their regular presence in marine parasites from several prominent groups.

10.3389/fmicb.2022.1113237

KEYWORDS

obligate blood-feeding, hematophagous, symbiosis, marine leech, parasitic isopod, parasitic copepod, *Vibrio*

Introduction

Hematophagy, or blood feeding, has emerged as an extremely successful nutritional strategy in thousands of species of animals from across the animal tree of life (Husnik, 2018). Considerable efforts have been made to understand the importance of these parasites in host vertebrate population dynamics, competition, energy flow and biodiversity (Holmes, 1996; Hudson et al., 2006; Wood et al., 2007). As a group, blood-feeding parasites are important influences on food webs and ecosystem health by both directly affecting the fitness and abundance of their immediate host, and also aiding the survival of predators of those species by increasing prey susceptibility to predation (Dunne et al., 2013). Obligate blood-feeding (OBF) invertebrates display a diversity of adaptations to accommodate blood feeding, including modifications of behavior, morphology, biochemistry and microbiology. Behaviorally, they locate and attach to their host prey using specific sensory mechanisms and questing-like behavior (Chaisson and Hallem, 2012). Morphologically, they often possess hooks and suckers, and highly extensible digestive systems, as well as sturdy body walls, to withstand extreme expansion during blood consumption (Khan, 1982; Sawyer, 1986; Graf, 2002). Because of this, OBF species can take large blood meals, many times their body weight, feed infrequently (e.g., every 6–12 months), and rely on comparably long digestion times (e.g., up to 14 days; Zebe et al., 1986; Lane, 1991). Biochemically, they produce vasodilators, anesthetics and anticoagulants to relieve venous constriction and ensure blood flow from their vertebrate hosts (Ribeiro, 1987). Finally, OBF taxa must contend with vitamin deficiencies (especially B vitamins), red blood cells that are difficult to digest, and heme toxicity (Toh et al., 2010). To overcome these dietary hurdles, they partner with internal bacteria that are believed to play an important role in counteracting the low digestibility and vitamin B deficiency, specifically.

Beneficial attributes of internal bacteria are generally wide ranging, from protection from predation or abiotic stresses to the dietary provision of missing nutrients or breakdown of foodstuffs. The possibility that internal bacteria could compensate for an unbalanced and difficult-to-digest blood meal was initially supported by research in the mid-1900s on medicinal leeches (see Jennings and Van Der Lande, 1967 and references therein). For terrestrial OBF species (i.e., tsetse fly, vampire bat), an alliance with bacteria appears to be necessary for animals to occupy the unusual niche of solely relying on blood for nutrition. Many studies since have provided evidence for high mortality rates of OBF species when deprived of their gut microbes (Lake and Friend, 1968; Rio et al., 2016), indicating a symbiotic relationship with bacteria as key to their success. Although experimental evidence is still limited, provisioning of B vitamins by bacterial symbionts has been demonstrated for blood-feeding arthropods, including tsetse flies and ticks (Sang, 1956; Pais et al., 2008). This has been investigated using genomic techniques, with evidence that bacterial symbionts found in the midgut of tsetse flies not only compensate for nutritional deficiencies of the invertebrate host, but also possibly complement other co-occurring resident bacteria (Akman et al., 2002; Toh et al., 2006; Snyder et al., 2010). In this way, persistent bacterial presence is believed to play an important role in parasite adaptation to blood-feeding, a specific niche that is generally unavailable to most animals.

Numerous OBF invertebrates also thrive in the marine realm, parasitizing bony and cartilaginous fish in the worlds' oceans. These include leeches, crustaceans, nematodes, and flatworms, to name a few. Like their terrestrial relatives, marine OBF species negatively influence their hosts, causing anemia, blindness, decreased reproductive fitness, and mortality, especially in bony fish populations, depending on the age of the fish and the number of infecting parasites (Kabata, 1981; Khan, 2012). For marine OBF species, an alliance with bacteria is also expected, but has so far not been well studied. For example, the Piscicolidae is a large family of marine leeches comprising ~60 genera and 200 species, yet only a single study documents a possible relationship with internal bacteria (Goffredi et al., 2012). This group of marine leeches is a likely candidate for the most ancestral clade within the annelid subclass Hirudinea (Utevsky et al., 2007), thus the investigation of this family is generally important for determining whether bacterial-mediated digestion and provisioning of complementary nutrients might be a key innovation in all blood-feeding leeches. Additionally, marine isopods within the family Cymothoidae include numerous species with a unique parasitic lifestyle—the exclusive consumption of vertebrate blood from the gills of primarily bony fish (Brusca, 1978, 1981). So far there have been very few studies related to the feeding biology of cymothoid isopods, from the perspectives of functional morphology of mouthparts or possible bacterial symbioses (Nagler and Haug, 2016). As with the piscicolids, there is a single publication on the possible relationship between bacteria and a species of blood-feeding Antarctic isopod (*Gnathia calva*; Juilfs and Wägele, 1987). Finally, the large and diverse copepod order Siphonostomatoida (> ~1,000 species) parasitize shallow- and deep-water fish worldwide, including farmed salmon and cod (Perkins, 1983). These parasites can consume so much blood that they negatively affect fish fitness, competition, and reproduction (Kabata, 1979; Godwin et al., 2015), thereby impacting not only natural ecosystems, but fish stock production for human consumption. Despite their omnipresence in the ocean, siphonostomatid

copepods remain entirely unexplored with regard to symbiotic partnerships.

Possible alliances between beneficial bacteria and obligate blood-feeding marine invertebrates were examined for two dominant categories of known blood-feeding marine invertebrates (leeches and crustaceans), collected from southern California coastal waters. Using DNA sequencing analysis combined with fluorescence microscopy, and bacterial cultivation, we document the prevalence of bacteria in 6 marine OBF species; fish and shark leeches (e.g., *Pterobdella, Ostreobdella*, and *Branchellion*), the isopods *Elthusa* and *Nerocila*, and the copepod *Lernanthropus* (Figure 1). Blood-feeding animals are not only important to study because of their potential symbiotic relationships with microbes, but because of their ability to act as both vectors for pathogens and the harm they cause to fish stocks. Surveying the microbial communities associated with marine blood-feeding invertebrates not only increases knowledge about nested biological diversity in the ocean, but may also provide insight into their successful nutritional strategy of parasitizing marine vertebrates.

Materials and methods

Sample collection

A variety of fish and elasmobranchs were collected from the coastal waters of southern California, either by hand or *via* trawl onboard expeditions in collaboration with the Orange County Sanitation District (OCSD)—Environmental Laboratory and Ocean Monitoring team. In all cases, permits to collect the fish hosts, from which we removed invertebrate parasites and, in most cases released, were held by R.A. (SC-13105), S.G. (SC-10578), and S-190710005-22077-001. The leech *Pterobdella occidentalis* (Goffredi et al., in press) was collected *via* minnow traps mainly from longjaw mudsuckers (*Gillichthys mirabilis*), plus a few additional host fish (Supplementary Table S1), while *Branchellion lobata* Moore 1952 was collected from various ray species, by beach seine, including primarily the Pacific round ray (*Urobatis halleri*) and bat rays (*Myliobatis californica*) in trawls off of Los Angeles or in San Diego Bay. The leech *Ostreobdella californiana* Burreson et al., 2019 was provided by Freeland Dunker, a veterinarian at the California Academy of Sciences. The species was originally described from rockfishes in the genus *Sebastes* in public display tanks in the Steinhart Aquarium, California Academy of Sciences, San Francisco, CA, although it has also been observed in nature on *Sebastes* from the San Francisco and Monterey Bay areas (Burreson et al., 2019). Marine isopods within the family Cymothoidae, including *Elthusa vulgaris* Stimpson, 1857 and *Nerocila californica* Schioedte and Meinert, 1881 were collected from various bony fish hosts, including Pacific sanddabs (*Citharichthys sordidus*) or killifish (*Fundulus parvipinnis*), by either beach seine or trawls. In some cases, the host fish was unknown, since cymothoids often detach from their host fish in trawls or seines (Brusca, 1978). The marine copepod *Lernanthropus latis* Yamaguti, 1954 (order Siphonostomatoida) was collected primarily from California corbina (*Menticirrhus undulatus*) by hook and line. Additionally, eggs were taken from *Elthusa* marsupium and egg masses were excised from adult *Lernanthropus* ($n = 3$ each). *Pterobdella* cocoons ($n = 3$ pooled collections) were recovered from surfaces of the collection vials, while in captivity. Specimens for molecular analysis were preserved within 2 h of collection in ~90% ethanol and stored at 4°C.

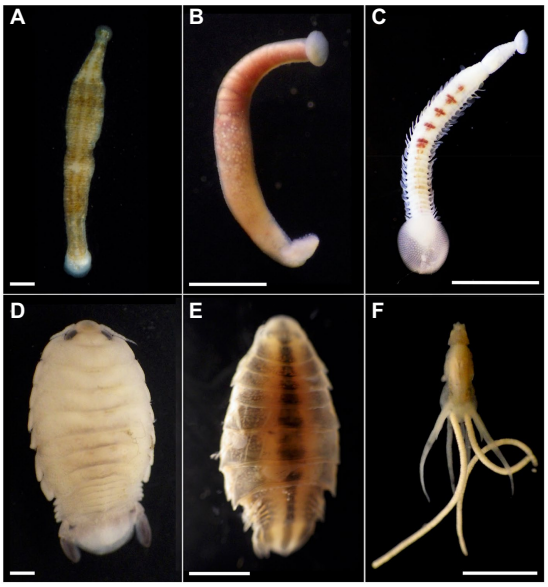

FIGURE 1
Marine blood-feeding invertebrates examined in this study. The bony fish leeches **(A)** *Pterobdella occidentalis* and **(B)** *Ostreobdella californiana*. Scale bars 1 mm. **(C)** The elasmobranch leech *Branchellion lobata*. Scale bar 5 mm. **(D)** The isopods *Elthusa vulgaris* and **(E)** *Nerocila californica*. Scale bars 2 mm. **(F)** The copepod *Lernanthropus latis*. Scale bar 4 mm. Photo credits: S. Goffredi.

Non-blood feeding crustaceans, including sea lice within the Caligidae (*Trebius* and *Lepeophtheirus*), were collected from the external surfaces of Pacific Round rays (*U. halleri*) and the California skate (*Raja inornata*), with *Clausidium vancouverensis* removed from the ghost shrimp (*Neotrypaea californiensis*). Ghost shrimp exoskeletons were also analyzed, as were swabs of skin mucus collected *via* sterile cotton swab from host fish. Swabs were stored at −80°C prior to molecular analysis. Seawater samples were taken from three collection locations and filtered (2 l) onto a 0.22 µm Sterivex-GP polyethersulfone filter (Millipore-Sigma, St. Louis, MO, United States) and stored at -80°C until DNA analysis.

Bacterial cultivation

Bacteria from the digestive systems of living specimens of *Branchellion*, *Pterobdella*, *Elthusa* and *Lernanthropus* were cultivated by plating homogenate (in 3X phosphate buffered saline

using a ground glass tissue homogenizer) on Difco-BD Marine Agar 2,216. Plates were incubated at 25°C for 1–3 days under aerobic conditions, and single colonies were further purified by standard T-streak. Cells were suspended in alkaline PEG (60 g of PEG 200 with 0.93 ml of 2 M KOH and 39 ml of water). This suspension was then heated to 96°C for 20 min in order to lyse the bacterial cells and liberate the DNA. 16S rRNA gene was then amplified using the general primers 27F and 1492R (Lane, 1991), with an anneal temperature of 54°C. Successful 16S rRNA gene products were sequenced at Laragen, Inc. (Culver City, CA). Hemolysis activity was assessed qualitatively *via* zone of lysis on blood agar plates (10% blood in Tryptic Soy Agar; Supplementary Figure S1). Bacteria capable of hemolysis created an obvious zone of depletion, at 25°C. Individual cell morphology was determined *via* scanning electron microscopy (SEM; Supplementary Figure S1). Bacterial cells for SEM were initially fixed in 3% glutaraldehyde in 0.1 mol L-1 cacodylate for 72 h at 4°C. Samples were then pulled onto a 0.22 µm polycarbonate filter (Millipore, Billerica, MA), washed in a graded ethanol series (50%, 75%, and 100%) and placed in hexamethyldisilazane for 1 h at room temperature. Filters were then mounted, palladium-coated (Hummer VI, Union City, CA), and visualized using a Phenom desktop SEM (FEI Instruments, Hillsboro, OR).

DNA extraction and 16S rRNA gene sequencing

Total genomic DNA was extracted from specimens, that had been rinsed in ethanol and dried, using the Qiagen DNeasy kit (Qiagen, Valencia, CA, United States) according to the manufacturer's instructions. Swab and filter extractions deviated from manufacturer's instructions by doubling reagent volumes during the initial lysis and incubation step (i.e., 360 µl ATL buffer and 40 µl Proteinase K) to cover the entire swab or filter area. The V4-V5 region of the 16S rRNA gene was amplified using bacterial primers with Illumina (San Diego, CA, United States) adapters on the 5′ end 515F (5′-TCGTCGGCAGCGTCAGA-TGTGTATAAG AGACAGGTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GTCT CGTG-GGCTCGGAGATGTGTATAAGAGACAGGGACTACHV GGGTWTCTAAT-3′; Caporaso et al., 2011). Note: Eight swabs and one non-blood-feeding isopod were not amplifiable for the 16S rRNA gene. The PCR reaction mix was set up in duplicate for each sample with Q5 Hot Start High-Fidelity 2x Master Mix (New England Biolabs, Ipswich, MA, United States) and annealing conditions of 54°C for 25 cycles. Duplicate PCR samples were then pooled, and 2.5 µl of each product was barcoded with Illumina NexteraXT index 2 Primers that include unique 8-bp barcodes (P55′-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXX-TCGTCG GCAGCGTC-3′ and P7 5′-CAAGCAGAAGACGGCATA CGAGAT-XXXXXXXX-GTCTCGTGGGCTCGG-3′). Secondary amplification with barcoded primers used conditions of 66°C annealing temperature and 10 cycles. Products were purified using Millipore-Sigma (St. Louis, MO, United States) MultiScreen Plate MSNU03010 with a vacuum manifold and quantified using Thermo Fisher Scientific (Waltham, MA, United States) QuantIT PicoGreen dsDNA Assay Kit on a BioRad CFX96 Touch Real-Time PCR Detection System. Barcoded samples were combined in equimolar amounts into a single tube and purified with Qiagen PCR Purification Kit 28,104 before submission to Laragen (Culver City, CA, United States) for 2 × 250 bp paired end analysis on the Illumina MiSeq platform with PhiX addition of 20%.

Amplicon sequence data was processed in Quantitative Insights Into Microbial Ecology (v1.8.0). Raw sequence pairs were joined and quality-trimmed using the default parameters in QIIME. Sequences were clustered with 99% similarity using the UCLUST open reference clustering protocol, and then, the most abundant sequence was chosen as a representative for each. Taxonomic identification for each representative sequence was assigned using the Silva-138 database, and checked *via* BLAST. Quantification and statistical analyses are described in the Results sections and figure legends. Comparisons were performed using ANOVA and statistical significance was declared at $p < 0.05$. Non-metric multidimensional scaling ordination (NMDS), analysis of similarity (ANOSIM), and similarity percentage analysis (SIMPER) analyses were performed in Primer-E, after square-root transforming the dataset and calculating Bray–Curtis similarities (Clarke and Warwick, 2001). The raw Illumina 16S rRNA gene barcode sequences and metadata collected in this study are available from the NCBI Small Read Archive (BioProject # PRJNA910167). The processed sequence data, as well as representative sequences, are available on the Dryad Digital Repository URL https://doi.org/10.5061/dryad.vmcvdncx9.

For at least one specimen from each OBF taxon, a 16S rRNA gene clone library was generated using the general primers 27F and 1492R (Lane, 1991) and the TOPO-TA kit (ThermoFisher, Waltham, MA, United States), according to the manufacturer's instructions. In this way, longer sequences of the 16S rRNA gene were recovered for nearly all dominant bacterial ribotypes (*via* Laragen, Inc.; Supplementary Figure S1). Longer sequences were assembled using Sequencher v4.10.1 (GeneCodes Corp., Ann Arbor, MI, United States) and trees were generated using Geneious Prime v2022.2.1 (Biomatters, Inc. San Diego, CA, United States). 16S rRNA sequences for clones and bacterial isolates are available from GenBank under accession numbers OP981048-OP981071.

Fluorescence *in situ* hybridization microscopy

Specimens for fluorescence *in situ* hybridization (FISH) microscopy were initially preserved in 4% sucrose-buffered paraformaldehyde (PFA) and kept at 4°C for 24–48 h. These PFA-preserved specimens were rinsed with 2× PBS, transferred to 70% ethanol, and stored at −20°C. Tissues were dissected and embedded in Steedman's wax (1 part cetyl alcohol: 9 parts polyethylene glycol (400) distearate, mixed at 60°C). An ethanol: wax gradient of 3:1, 2:1 and 1:1, and eventually 100% resin, was

used to embed the samples (1 h each treatment). Embedded samples were sectioned at 3 µm thickness using a Leica RM2125 microtome and placed on Superfrost Plus slides. Sections were dewaxed in 100% ethanol rinses. As a reference, some sections were histologically examined *via* the Wright stain (2.5 min exposure) and visualized. To specifically target the associated OBF *Vibrio*, a probe that was an exact match was used (*Vibrio* GV; 5′-AGGCCACAACCTCCAAGTAG-3′; Giuliano et al., 1999), labeled with the fluorochrome Cy3 at both the 3′ and 5′ terminus. For universal detection of most bacterial 16S rRNA genes, we used the probe EUB338 (Amann et al., 1990). The full comprehensive EUB338 probe set was not employed since probes EUB338-II and EUB338-III target Planctomycetales and Verrucomicrobiales, respectively, and 16S rRNA genes from these specific bacteria were not recovered *via* barcoding efforts. A nonsense probe (*NonEub*; 5′-ACTCCTACGGGAGGCAGC-3′) was used as a negative control. Hybridization buffers and wash buffers were prepared according to Pernthaler and Pernthaler (2005). The samples were incubated in hybridization buffer containing 50 nM probe at 46°C for 4–8 h, followed by a 15 min wash at 48°C. Sections were counterstained with 4′6′-diamidino-2-phenylindole (DAPI, 5 mg/ml) for 1 min, rinsed and mounted in Citifluor. Tissues were examined by epifluorescence microscopy using either a Nikon E80i epifluorescence microscope with a Nikon DS-Qi1Mc high-sensitivity monochrome digital camera or a Zeiss Elyra microscope with an ANDOR-iXon EMCCD camera. No FISH microscopy was conducted on *Ostreobdella*, due to insufficient initial preservation of the specimens, or *Nerocila*, due to low sample sizes.

Transmission electron microscopy

A single *Pterobdella* specimen for TEM microscopy was initially preserved in 3% glutaraldehyde buffered with 0.1 M phosphate and 0.3 M sucrose (pH 7.8). Samples were washed in 0.1 M sodium cacodylate with 24% sucrose and post-fixed with 1% OsO4 in 0.1 M sodium cacodylate for 1 h. Samples were stained in 3% uranyl acetate in 0.1 M sodium acetate buffer for 1 h, dehydrated in ethanol and embedded in Spurr's resin. Thick sections (0.4 µm) were stained with methylene blue and examined using a Nikon E80i microscope, while thin sections (70 nm) were stained with lead citrate and examined using a Zeiss EM 109 transmission electron microscope.

Results and discussion

The microbiomes of obligate blood feeding marine invertebrates

The microbiome was characterized for a number of diverse obligate blood-feeding marine invertebrate taxa, including the fish leeches *Pterobdella occidentalis* and *Ostreobdella californiana*, the elasmobranch leech *Branchellion lobata,* the copepod *Lernanthropus latis*, and two isopod species, *Elthusa vulgaris* and *Nerocila californica*, the latter of which might also feed on tissue, in addition to blood (Bunkley-Williams and Williams, 1998; Figure 1; Supplementary Table S1). Bacterial 16S rRNA gene amplicon barcoding revealed that the microbiome of the OBF invertebrates was significantly distinct from non-blood-feeding relatives (in the case of crustaceans; ANOSIM $R = 0.54$, $p = 0.01$), swabs of fish prey species (in the case of the leeches; ANOSIM $R = 0.43$, $p = 0.01$), and surrounding seawater (ANOSIM $R > 0.65$, $p = 0.01$ for both; Figure 2; Supplementary Table S2). Blood-feeding invertebrate species had a significantly lower microbiome diversity (average Shannon index for each species was 0.5–1.5, at the 99% similarity 16S rRNA gene level; Supplementary Table S1) compared to non-blood-feeding invertebrates, swabs of fish prey species, and surrounding seawater (average Shannon index of 1.9–2.5; ANOVA $p < 0.0001$). In past studies, comparatively lower microbial diversity has been observed for other OBF taxa, including the freshwater leech genera *Macrobdella* and *Hirudo* (Graf et al., 2006; McClure et al., 2021). One exception in this study was the copepod *Lernanthropus* with a slightly higher diversity (average Shannon index of 1.8 ± 0.4; Supplementary Table S1), comparable to the least diverse environmental sample. In all cases, the microbial community structure of each of the 6 obligate blood-feeding marine species was unique (ANOSIM $R > 0.8$, $p = 0.01$) and remained stable over 3–5 years of collection (Figure 2F; Supplementary Table S2), suggesting specific and non-transient associations.

Blood-feeding invertebrate species were mostly dominated by only a few bacterial groups. In nearly all OBF species, ribotypes within the Vibrionaceae (*Vibrio*, *Alivibrio, Photobacterium*) were the most dominant member of the microbiome, comprising 39–81% of the total recovered 16S rRNA gene sequences, on average per OBF taxon (described in more detail below; Figure 2; Supplementary Figure S1; Supplementary Table S3). This was significantly higher than for non-blood feeding invertebrates and swab surfaces, both of which also hosted *Vibrio* (15%–18% of average recovered ribotypes per comparison group; ANOVA $p = 0.0004$ for OBF crustaceans versus non-blood feeders; ANOVA $p = 0.00003$ for OBF leeches versus fish swabs; Figures 2, 3; Supplementary Table S3). By contrast, seawater samples contained <2% *Vibrio*, based on recovered 16S rRNA genes (Figures 2, 3; Supplementary Table S3). The second most prominent associated bacterial ribotype depended on the specific OBF marine taxa, and included an unidentified member of the Flavobacteriaceae (22% on average recovered from *Ostreobdella*), an unidentified member of the Porticoccaceae (23% on average for *Lernanthropus*), *Shewanella* (21% on average for the *Elthusa*), and an unidentified member of the Oceanospirillaceae (34% average 16S rRNA gene sequences recovered from *Pterobdella*; Figure 2; Supplementary Table S3). For *Branchellion*, an undescribed gammaproteobacteria and betaproteobacteria co-occurred with *Vibrio*, each comprising ~15%–17% of recovered ribotypes (Figure 2; Supplementary Table S3). The maintenance of a gut community dominated by only 2–3 bacterial taxa has been observed for both *Macrobdella* and numerous hirudinid leeches

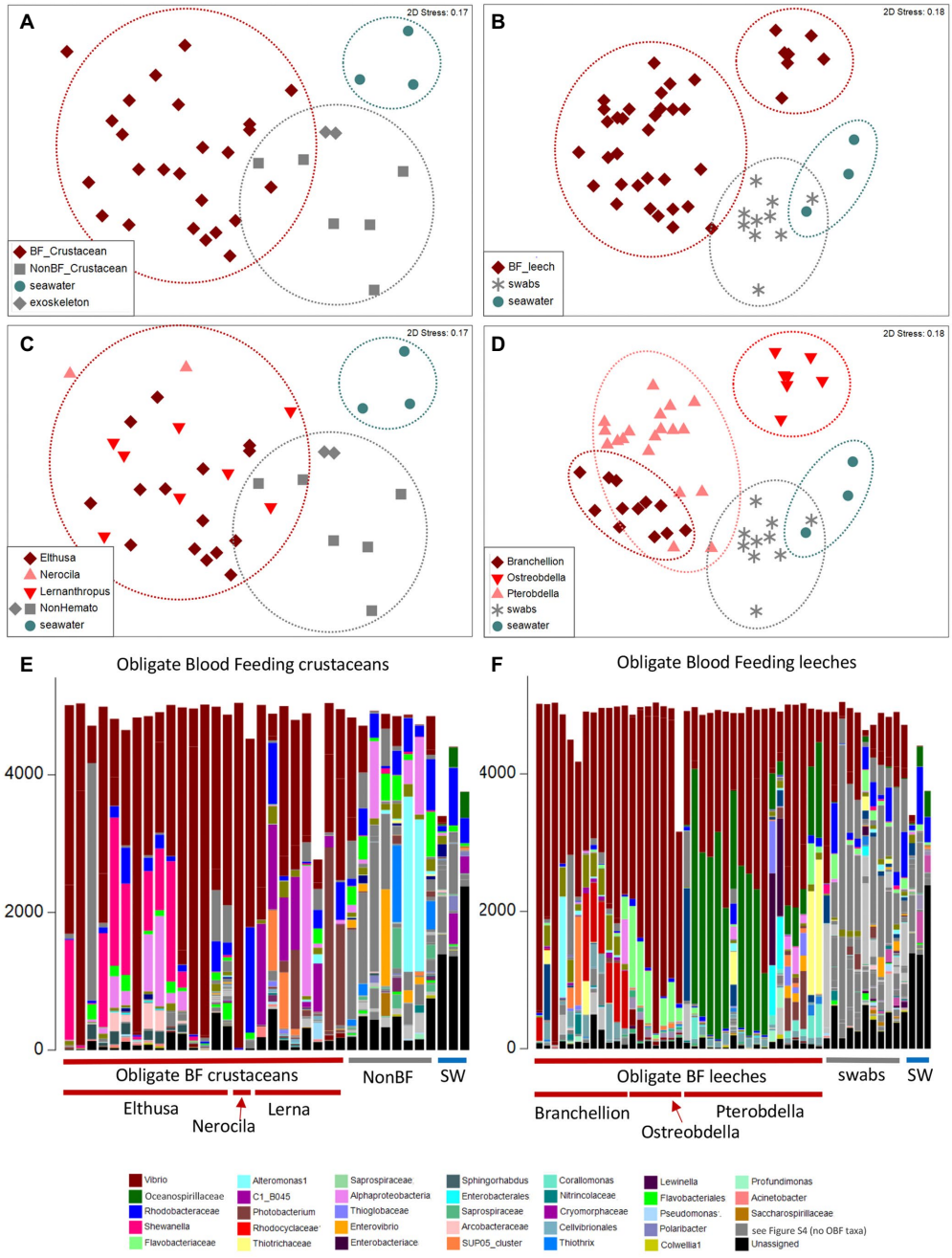

FIGURE 2
Microbiome diversity analysis of obligate blood feeding (OBF) crustaceans and leeches, based on 16S rRNA gene sequence similarity, using Non-metric multidimensional scaling (NMDS) ordination plots based on Bray–Curtis similarity resemblance, at the **(A,B)** broad category level, including blood-feeding versus comparison samples of non-blood feeding (NonBF) taxa, biological surfaces, and seawater (SW) and at the **(C,D)** specific blood-feeding taxa level. **(E,F)** Relative abundance of bacterial community structure at the genus level, from marine blood-feeders collected primarily from southern California coastal waters, including isopods *Elthusa* and *Nerocila*, the copepod *Lernanthropus* (Lerna), and leeches *Branchellion, Ostreobdella,* and *Pterobdella* (specific specimens are listed in Supplementary Table S1 in the same order as shown in the bar charts). Assigned bacterial taxa are color-coded as shown below. Taxa in dark gray were only found in the non-blood feeder (NBF) or seawater (SW) samples. Taxa in light gray were minor taxa in all specimens. See Supplementary Table S4 for a full key.

(Graf et al., 2006; Kikuchi and Graf, 2007; Laufer et al., 2008; McClure et al., 2021). Many of the undescribed secondary bacterial ribotypes associated with the marine OBF taxa were dissimilar from known bacteria (< 90% 16S rRNA gene identity; Supplementary Figure S2), thus likely representing novel taxa. Some, however, including the Flavobacteriaceae and Porticoccaceae are known to associate with marine invertebrates (Garren et al., 2009; Webster et al., 2013; Dishaw et al., 2014),

making them interesting candidates for follow-up studies on invertebrate endemic microbiome members. Within the marine OBF taxa, occasional bacteria and those present in much lower numbers, likely represent transient organisms, as observed in the medicinal leech (Worthen et al., 2006). For example, one *Nerocila* specimen revealed a near singular dominance of *Vibrio* (99% of ribotypes), whereas the other *Nerocila* specimen hosted both *Vibrio* and an unidentified member of the Rhodobacteraceae (22% of ribotypes), which was also found in appreciable numbers in *Elthusa*, non-blood feeding relatives, swabs, and seawater (>10% of ribotypes; Figure 2; Supplementary Table S3).

By comparison to the marine OBF taxa, very few bacterial ribotypes accounted for more than 10% of recovered sequences for the non-blood-feeding crustaceans, ghost shrimp exoskeletons, and swabs from fish skin (based on 99% 16S rRNA gene similarity). For those that did, some were shared with the OBF marine taxa, including *Pseudoalteromonas*, an undescribed alphaproteobacteria, and the aforementioned *Vibrio* and Rhodobacteraceae (Figure 2; Supplementary Table S3). Various other notable bacterial groups recovered from the environmental and non-blood-feeding samples were either not observed in the marine OBF taxa or were at very low levels, including *Alteromonas, Thiothrix, Rubitalea, Colwellia*, and *Marinomonas*, to name a few (Figure 2; Supplementary Table S3). This suggests a distinct, and winnowed, bacterial community associated with the marine OBF taxa compared to biological surfaces of invertebrates and vertebrates, non-blood feeding crustaceans, and seawater. This pattern is similar to previous reports of marine bacterial communities, which are distinctly reduced when associated with an animal host (Fraune and Bosch, 2007; Goffredi et al., 2021). Additionally, unassigned 16S rRNA gene sequences comprised 45% for the seawater samples and 9% for the non-blood-feeding taxa, compared to only 1–5% of the average 16S rRNA genes recovered from each marine OBF taxon (ANOVA $p < 0.003$).

Microbiome comparisons between lifestages differed for the various obligate blood feeding taxa. Eggs taken from the *Elthusa* marsupium and egg masses excised from adult *Lernanthropus* had very similar microbial communities to the adults, suggesting possible transmission of bacteria from parent to offspring (n = 3 each; ANOSIM $R < 0.225$, $p > 0.14$). Although additional fluorescent microscopy on the eggs would add additional evidence for vertical transmission, the similarities *via* 16SrRNA gene recovery included the community evenness of these specific bacterial taxa; *Vibrio* and *Shewenalla* in the case of *Elthusa* and *Vibrio* and Porticococcaceae for *Lernanthropus* (Supplementary Figure S3). The main difference from the adults was the significant occurrence of *Pseudoalteromonas* on the eggs of both species, which has been seen for other crustaceans (Gil-Turnes et al., 1989) and natural marine surfaces more broadly (Skovhus et al., 2007; Moisander et al., 2015). In crustaceans, the act of egg brooding has been shown to facilitate transfer of bacteria pseudovertically from parent to offspring (Goffredi et al., 2014; Sison-Mangus et al., 2015). *Pterobdella* cocoons, on the other hand, had very dissimilar microbiomes from the adults (n = 3 pooled collections; ANOSIM $R = 0.98$, $p = 0.003$), with comparatively high diversity values (avg $H' = 2.98$, vs. 1.20 for the adults). Although cocoons could not be tracked to a specific parent, since they were found already deposited on surfaces, the microbial community demonstrated a specific reduction in *Vibrio*, a complete lack of the Oceanospirillaceae, and the presence of Rhodobacteraceae and numerous other groups not observed in the adults (Supplementary Figure S3). In leeches, some symbionts are thought to be transmitted vertically (Kikuchi and Fukatsu, 2002), for example in eggs that are carried by a brooding parent (Kikuchi and Fukatsu, 2002), while others, especially those in the crop that can be cultured in the lab, are suspected to be acquired from the environment, of either the cocoon or hatchlings.

Consistent and distinct *Vibrio* associated with obligate marine blood feeders

As noted, bacteria within the *Vibrio* genus dominated the tissues of six phylogenetically-diverse OBF marine taxa, including leeches, isopods, and copepods. The most common *Vibrio* ribotypes, based on 16S rRNA gene sequencing, generally grouped into 4 *Vibrio* species clades, most closely related to *Vibrio tasmaniensis, V. parahaemolyticus/V. alginolyticus, V. anguillarum* and *V. atypicus*, previously recovered from environmental sources (Supplementary Figure S1). Out of a total of 135 distinct Vibrionaceae 16S rRNA gene amplicons recovered from all samples (based on 99% similarity), seven were dominant and accounted for 80% of the total Vibrionaceae diversity. These *Vibrio*-specific amplicons were either exclusive to the marine OBF specimens, or were present in much higher abundance, than the non-blood-feeding invertebrates and other nearby biological surfaces (Figure 3). For two of the blood-feeding taxa, identical *Vibrio* ribotypes were associated with specimens collected on different prey fish (e.g., *Pterobdella* from the longjaw mudsucker and goby, and *Elthusa* from killifish vs. Pacific sanddabs). Even the single specimen of *Pterobdella abditovesiculata* from the Hawaiian host fish *Eleotris* associated with the same *Vibrio* ribotype as those found in *Pterobdella occidentalis* from southern California.

Vibrionaceae species are well-known to associate, somewhat ubiquitously, with biological surfaces and plankton in the oceans (Montanari et al., 1999; Romalde et al., 2014). Although also known to degrade chitin and commonly associate with marine invertebrates, *Vibrio* species, in other studies, are mostly generalists and show little host preference (Preheim et al., 2011). However, some specific and persistent associations with beneficial Vibrionaceae have been detailed in other well-known systems, including the Hawaii bobtail squid and luminous fishes (Haygood and Distel, 1993; Visick et al., 2021). A specific association for the OBF marine taxa is suggested by evidence that the *Vibrio* ribotypes recovered were generally not associated with related invertebrates and nearby biological surfaces (Figure 3). Further, *Vibrio* were extremely abundant in

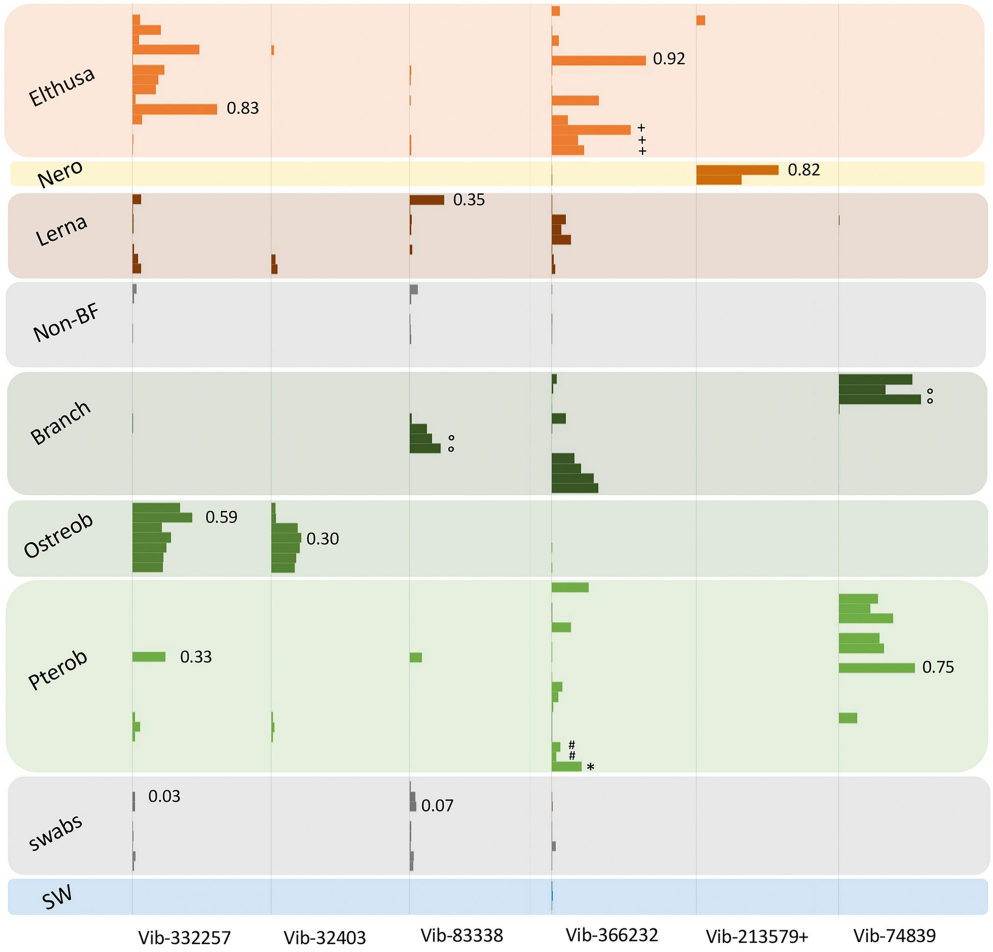

FIGURE 3
Relative abundances of the 7 most prevalent *Vibrio* ribotypes (based on 99% 16S rRNA gene sequence similarity) from obligate marine blood-feeders including the isopods *Elthusa* and *Nerocila* (Nero), copepod *Lernanthropus* (Lerna), and leeches *Branchellion, Ostreobdella,* and *Pterobdella,* compared to non-blood-feeding crustaceans (Non-BF), swabs of fish skin, and seawater (SW). Bars are scaled according to percent abundance of each *Vibrio* ribotype as a function of the entire microbial community in that specimen (for reference, occasional numbers indicate the % abundance of that bar). A total of 135 Vibrionaceae ribotypes were recovered from all samples in total, but the 7 portrayed here accounted for 80% of the total Vibrionaceae diversity. See Supplementary Figure S1 for the phylogenetic position of these ribotypes. Note the pooling of *Vibrio* ribotypes 19,850 with 213,579 (+) common only in the *Nerocila* specimens *via* amplicon sequencing. Occasional symbols note distinction within OBF taxa, including *Pterobdella occidentalis* recovered from the goby (#), and *P. abditovesiculata* from Hawaii (*), as well as *Branchellion* collected from rays other than the pacific ray (°), and *Elthusa* collected from killifish (+).

the microbiome of *Elthusa, Branchellion* and *Nerocila* (e.g., comprising >96% of the total bacterial community in some individuals). By contrast, *Vibrio* species were detected, but low, in the microbiome of three temperate copepod species from the Gulf of Maine, with the most abundant Vibrionaceae genus comprising only 4% of all gamma-proteobacterial sequences recovered (Moisander et al., 2015). Finally, while pathogenic marine *Vibrio* species can be common, they are usually prevalent in only 20%–50% of hosts sampled at a given time (Davis and Sizemore, 1982; Sullivan and Neigel, 2018). The prevalence of *Vibrio* in the marine OBF taxa surveyed in this study was 100% and the pattern of *Vibrio* dominance and community structure continued for individuals collected over many years, thereby reducing the possibility of a transient bacterial infection.

Since *Vibrio* was the most common bacterial group observed *via* 16S rRNA gene barcoding and cloning of OBF marine taxa, the ultrastructural position of *Vibrio* cells within specific tissues was examined (Figures 4–7; Supplementary Figure S4). Using specific fluorescence *in situ* hybridization microscopy, *Vibrio* cells were clearly observed within the lumen of the copepod *Lernanthropus*, among partially-digested bloodmeal (Figure 4). The morphology of siphonostomid copepods varies considerably depending on the precise attachment to their host fish (e.g., holdfast structures and abdominal lobes; Boxshall, 1986), thus we might expect to see a varied integration with *Vibrio*, or other bacterial associates,

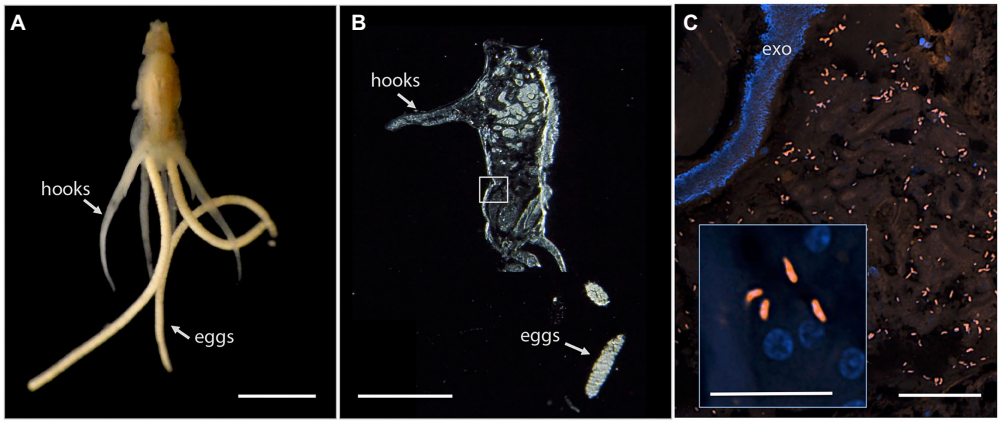

FIGURE 4
Fluorescent visualization and localization of *Vibrio* in **(A)** the parasitic copepod *Lernanthropus latis*, with clasping hooks and egg strings noted. Image taken with a Pentax WG-III handheld camera. Scale bar 2mm. **(B)** Cross section of the specimen after being embedded in Steedman's wax and sectioned. Scale bar 2mm. **(C)** A *Vibrio*-specific fluorescent probe revealed comma-shaped bacterial cells, shown in orange *via* Cy3, within the digestive lumen space among bloodmeal. Scale bar 20μm. (inset) A magnified view of the *Vibrio* cells, near copepod nuclei, shown in blue *via* DAPI. Scale bar 5μm. exo, autofluorescent exoskeleton.

depending on the species examined in the future. For *Elthusa* *Vibrio* cells were observed in the intestine lumen, in an area that appeared to be filled with blood based on the Wright stain, near a junction with the digestive ceca (Figures 5D,F). Note that during dissection, the entire *Elthusa* intestine was pulsing with blood, which evacuated through the mouth when pressure was applied. For the leeches *Branchellion* and *Pterobdella*, *Vibrio* cells were observed in large regions of the blood-filled crop, often in and among the obvious densely packed erythrocytes of the prey host (e.g., Figure 6D). From embedded *Branchellion* tissues, preliminary laser capture microdissection of these bacteria-containing regions, resulted in amplification of only *Vibrio* 16S rRNA gene from the captured tissue (Supplementary Figure S1). Similarly, crop tissues specifically excised from 5 *Pterobdella* specimens only yielded *Vibrio* 16S rRNA genes. The crop is the largest component of the leech digestive tract that stores the bloodmeal after ingestion. It is also the location of the symbionts detected in previous studies on hirudinid leeches (Graf et al., 2006; Worthen et al., 2006). Symbionts can be free in the lumen, like individual *Aeromonas* cells inside the medicinal leech crop, or attached as a biofilm to tissue epithelia, as in *Mucinivorans hirudinis* (Kikuchi and Graf, 2007; Nelson et al., 2015). Notably, a biofilm of bacteria, not identified as *Vibrio*, was observed in *Pterobdella* attached to the luminal epithelia of the unique paired mycetomes (or esophageal diverticula), possessed by this genus (Figure 7). Transmission electron (TEM) microscopy of these putative mycetomes revealed numerous bacteria-like cells, some dividing (Figure 7G). Mycetomes were excised from two *Pterobdella* specimens, and analyzed *via* direct 16S rRNA gene sequencing, revealing the singular presence the Oceanospirillaceae, suggesting that these bacteria reside in a location distinct from the co-occurring *Vibrio*. At least 3 leech genera within the family Glossiphoniidae possess diverse bacterial symbionts in esophageal organs (Kikuchi and Fukatsu, 2002; Perkins et al., 2005). It is novel, however, to observe a mycetome-associated symbiont in a member of the Piscicolidae (within the order Aryhnchobdellida; Graf et al., 2006). Bacteria have also been observed residing within nephridia and bladders of leech excretory systems (Graf et al., 2006; Kikuchi et al., 2009), however it is not currently known whether this occurs in marine OBF leeches as well. Finally, *Vibrio* cells within the digestive tissues in the marine OBF taxa were not only observed within the crop lumen, but also in association with what appeared to be hemocytes of the invertebrate parasite. For the isopod *Elthusa*, *Vibrio* cells were observed inside of cells, at a specific junction between the midgut and the anterior digestive ceca (Figure 5). Similarly, for *Branchellion*, *Vibrio* were not only among erythrocytes, but also intracellular in what appeared to be hemocytes given the obvious presence of multiple nuclei (Figures 6E,F). Hemocytes, which are invertebrate immune cells with a phagocytic function, are thought to play a major role in the establishment and maintenance of beneficial associations between bacteria and invertebrates. For example, in both the medicinal leech and the Hawaiian bobtail squid, hemocytes have been observed interacting with the resident bacterial community (Silver et al., 2007; McAnulty and Nyholm, 2016), suggesting that symbionts can modulate the cellular immune response of the host, and vice versa.

Beneficial bacteria are most often housed (or rather colonize and persist) in a particular tissue, and their position within the body of the host animal can give clues as to their particular role, if any. The position of *Vibrio* in and among the bloodmeal hints at a possible role in red blood cell digestion in the marine OBF examined in this study. *Vibrio* isolates cultivated from the tissues of *Branchellion*, *Pterobdella*, *Elthusa*, and *Lernanthropus* matched the most dominant 16S rRNA gene amplicons (Supplementary Figure S1). For all but one of these *Vibrio* isolates, an ability to effectively lyse vertebrate blood cells

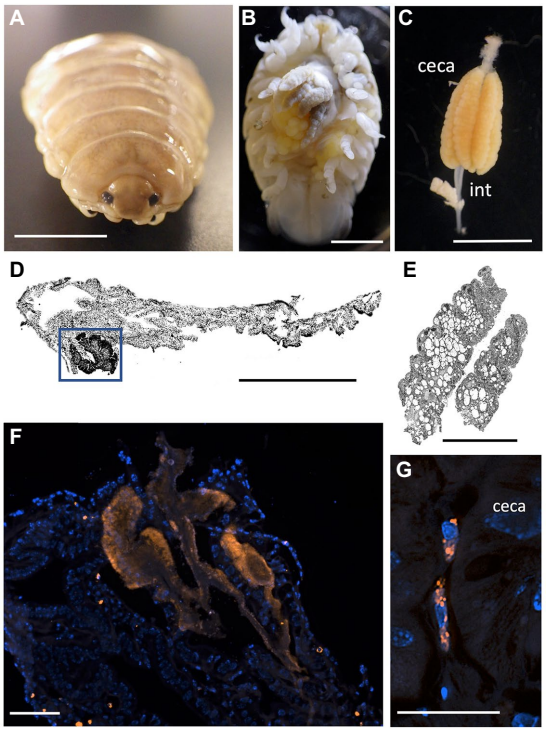

FIGURE 5
Fluorescent visualization and localization of *Vibrio* in **(A)** the parasitic isopod *Elthusa vulgaris*. **(B)** Ventral dissection showing the enlarged digestive ceca. Scale bar 5mm. **(C)** Excised digestive ceca and intestine. Scale bar, 4mm. A-C were taken with a Pentax WG-III handheld camera. **(D)** intestine sectioned and Wright stained. Scale bar, 1mm. Square indicates region in **F**. **(E)** ceca sectioned and Wright stained. Scale bar, 1mm. **(D,E)** Imaged *via* light microscopy. **(F)** A *Vibrio*-specific fluorescent probe, shown in orange with Cy3, revealed a strong signal within a darkly stained area of the intestine, near the ceca junction. Scale bar 100μm. **(G)** *Vibrio* cells, shown in orange, observed inside of cells, at a specific junction between the midgut and the anterior digestive ceca. Scale bar 20μm. Isopod nuclei are shown in blue, *via* DAPI. int., intestine.

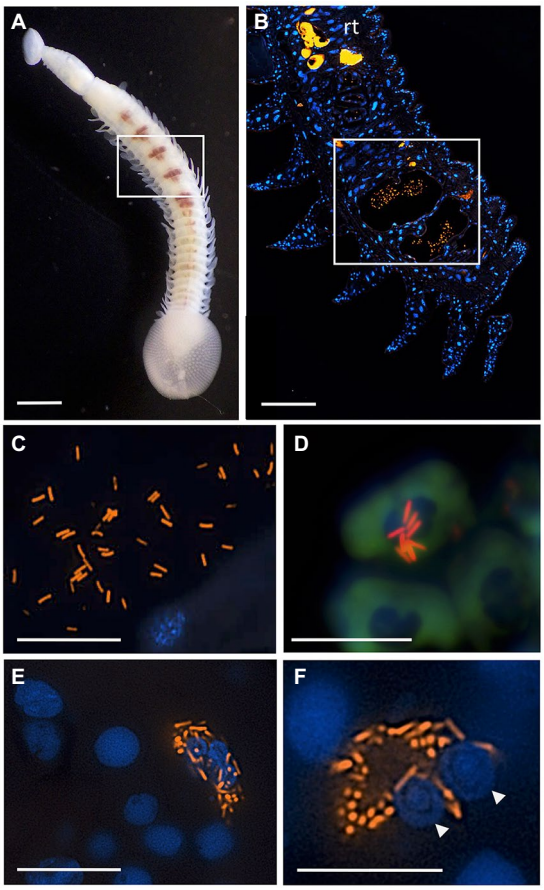

FIGURE 6
Fluorescent visualization and localization of *Vibrio* in **(A)** the elasmobranch leech *Branchellion lobata*. Square indicates regions of the crop filled with blood, as in **B**. Scale bar 1mm. Taken with a Pentax WG-III handheld camera. **(B)** A partial longitudinal section through an individual, showing fluorescent hybridization using a *Vibrio*-specific probe to illuminate bacteria cells (shown in orange, *via* Cy3), with leech cell nuclei shown in blue (*via* DAPI). Square indicates region in **C–F**. Scale bar 100μm. **(C,D)** A *Vibrio*-specific fluorescent probe revealed rod-shaped bacterial cells, shown in orange *via* Cy3, within the lumen space of the crop among bloodmeal (fish host blood cell shown in green, autofluorescence). **(D)** *Vibrio* cells appear to be on top of host cell and nucleus. **(E,F)** Within leech cells, sometimes in proximity to multiple nuclei (arrowheads), shown in blue *via* DAPI. **C-F** scale bars are 10μm. rt., autofluorescent reproductive tissues.

embedded in agar media was observed, providing evidence for this capability. Since red blood cells constitute the largest cellular component of blood, their efficient lysis is a central requirement for blood-feeding parasites. A similar ability to lyse erythrocytes, and subsequently establish in the leech gut, has been observed in *Aeromonas veronii*, the primary symbiont of *Hirudo verbana* (Maltz and Graf, 2011). Bacteria generally found in association with medicinal leeches are thought to play an important role in the specific digestion of the blood meal, as well as the detoxification of heme (Toh et al., 2010). The latter appears to be a significant, and possibly underestimated, obstacle for blood-feeding animals, given that heme can generate hydroxyl radicals and reactive oxygen species, capable of damaging proteins, lipids and DNA (Aft and Mueller, 1984). Other than initiating the digestion of erythrocytes, several other functions for the dominant crop bacteria have been proposed for hirudinid leeches, including providing essential nutrients to the host, prevention of other bacteria from colonizing the crop, and immunological priming of the host invertebrate (Graf, 2002; Maltz and Graf, 2011; Rio et al., 2016; Husnik, 2018). It is not yet known whether bacterial residents play an important role in overcoming the dietary hurdles of low digestibility and vitamin B deficiency in marine OBF taxa.

Conclusion

While most blood-feeding animals examined so far host internal bacterial symbionts that aid in some aspect of their nutrition, nearly all studies have focused on terrestrial

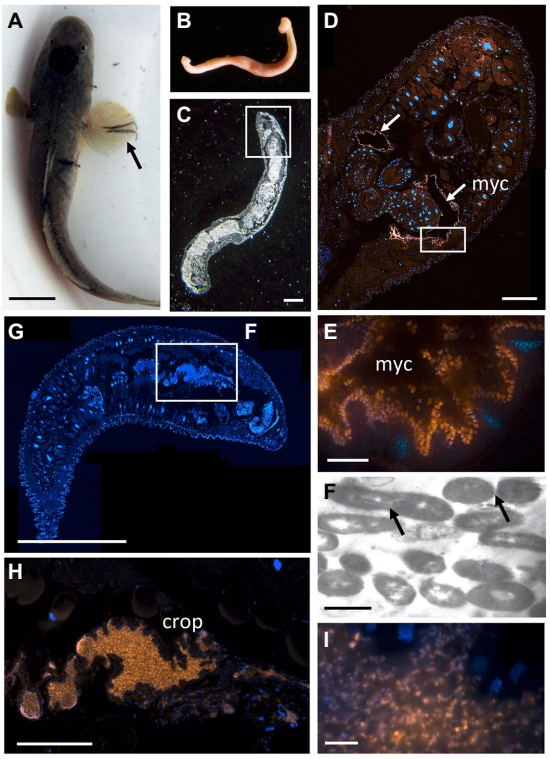

FIGURE 7
The bony fish leech *Pterobdella occidentalis* **(A)** on the fins and body of the longjaw mudsucker (*Gillichthys mirabilis*), indicated by the arrow. Scale bar 1cm. **(B)** Whole specimen, taken with a Pentax WG-III handheld camera. **(C)** Specimen embedded in Steedman's wax and sectioned, imaged *via* light microscopy. Square indicates region in **D**. Scale bar, 1mm. **(D)** Longitudinal section through the anterior end, showing general bacteria cells in a pair of mycetomes (shown in orange, *via* Eub338-Cy3), counterstained with DAPI, shown in blue. Square indicates region in **E**. Scale bar, 200μm. **(E)** Close-up of bacterial cells, shown in orange, attached to the putative mycetome epithelia. Scale bar is 10μm. **(F)** Transmission electron microscopy of a putative mycetome, revealing bacteria-like cells, some which appear to be dividing (arrows). Scale bar, 1μm. **(G)** Longitudinal section through a near complete leech specimen, stained with DAPI. Scale bar, 1mm. **(H)** *Vibrio*-specific cells within the crop (shown in orange, *via* Cy3), counterstained with DAPI, shown in blue. Scale bar, 200μm. **(I)**. Close-up of bacterial cells, shown in orange, within the crop. Scale bar, 10μm. myc, mycetomes.

blood-feeders. In this study, persistent internal associations with bacteria were observed for 6 phylogenetically-diverse species of marine obligate blood-feeders, including leeches, isopods, and copepods. These blood-feeding invertebrates possessed microbiomes of low diversity, mostly dominated by only a few bacterial groups, that were significantly distinct from non-blood-feeding relatives, biological surfaces, and seawater. Notably, *Vibrio* was abundant in all individuals examined and microscopy revealed their localization to the blood-filled lumen spaces. This hints at a possible evolutionary convergence of this bacterial genus as an essential abettor for a diet based solely on blood from marine vertebrates. We have preliminary indications of *Vibrio* presence in other hematophagous copepods, as well, including *Haemobaphes* and *Kroyeria* species, and it will be interesting to examine other marine OBF phyla, for example nematodes and flatworms, to see whether this prevalence extends even further across the parasite tree of life. Stable bacterial communities over 3–5 years of collection for each group examined in this study suggests intimate and non-random bacterial associations. Further investigations will require additional specimens and time points to understand the pervasiveness of the putative partnerships, and the specificity or degree of taxonomic diversity among possible bacterial partners, and additional life stages to uncover possible strategies for bacterial perpetuation. Eggs taken from the crustaceans *Elthusa* and *Lernanthropus* had very similar microbial communities to the adults, suggesting possible transmission of bacteria from parent to offspring in some hematophagous taxa. The role of the microbes associated with marine blood-feeding invertebrates is not yet determined, but there is every reason to believe that these animals face the same challenges with their exclusive subsistence on vertebrate blood. The distinctiveness of their bacteria from those associated with the environment and other non-hematophagous animals, further suggests a specific, exclusive, and likely functional relationship.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: Small Read Archive (PRJNA910167), GenBank (OP981048-OP981071), and https://doi.org/10.5061/dryad.vmcvdncx9.

Ethics statement

Fish and elasmobranchs were collected from the coastal waters of southern California, either by hook and line, beach seine, fish trap or *via* trawl onboard expeditions in collaboration with the Orange County Sanitation District (OCSD)—Environmental Laboratory and Ocean Monitoring team. In all cases, permits to collect the fish hosts, from which we removed invertebrate parasites and, in most cases released, were held by RA (SC-13105 and S-190710005-22077-001) and SG (SC-10578).

Author contributions

SG conceived the study, designed the research, performed molecular analyses, and wrote the paper, with contributions from SG, RA, RH, and JR. RA arranged collection of all samples and aided in our understanding of parasitic taxa. RH and JR performed the research, including amplicon sequencing analyses and

fluorescence microscopy in the laboratory. All authors contributed to the article and approved the submitted version.

Funding

Funding for this project was made possible by generous donations by Ron and Susan Hahn and a National Science Foundation grant to SG (IOS-1947309).

Acknowledgments

We thank A. Lizarraga, Bianca dal Bó, Norma Morella, Lyn Tam, Morgan Miller, and Pedro Castellanos for laboratory assistance. We also thank Occidental College undergraduates involved in the Spring 2017 and 2021 semesters of Microbial Symbiosis (Bio350) for some molecular and microscopy results. We would like to thank Danny Tang (Scientist, Orange County Sanitation District) and Julianne Passarelli (Exhibits and Collections Curator, Cabrillo Marine Aquarium) for their enthusiasm and expertise regarding parasitic copepods, Troy Sakihara (Hawaii Division of Aquatic Resources, Hilo, Hawaii) for providing *Pterobdella abditovesiculata* from *Eleotris sandwicensis*, and Carin Latino (Cabrillo Marine Aquarium) for aiding with holding of round stingrays. Coastal access for collection of parasites was provided by Martin Ruane (U.S. Navy, Mugu Naval Air Station), Jill Terp (U.S. Fish and Wildlife Service) and Jeff McGovern (U.S. Navy; Seal Beach National Wildlife Refuge), Tim Dillingham (CDFW, San Diego Marine Protected Areas), Eileen Maher (Port of San Diego), and Brian Collins (USFWS, South San Diego Bay National Wildlife Refuge).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1113237/full#supplementary-material

SUPPLEMENTARY TABLE S1
Specimens analyzed in this study, showing specimen ID, collection year, location, and host vertebrate, listed in the same order as in the stacked bar charts in Figures 2E,F.

SUPPLEMENTARY TABLE S2
Results from pairwise one-way Analysis of Similarities (ANOSIM) analysis for all comparisons mentioned in the text.

SUPPLEMENTARY TABLE S3
Microbial taxa that contribute up to 70% of the community structure of the microbiome (based on 16S rRNA genes amplicons) for obligate blood feeding (OBF) leeches and crustaceans, and comparison environmental samples. Highlighted in green are the most common bacterial groups in the OBF taxa. Those highlighted in gray are the most common bacterial groups in the comparison samples (non-blood feeding relatives, biological surfaces, and seawater). As well as the Shannon index calculated based on bacterial 16S rRNA gene sequences recovered from barcoding.

SUPPLEMENTARY TABLE S4
A complete color key for Figures 2E,F, showing the relative abundance of bacterial community structure at the genus level, for marine blood-feeders examined in this study.

SUPPLEMENTARY FIGURE S1
Phylogenetic relationships among *Vibrio* associated with blood feeding marine crustaceans and leeches, relative to selected cultured representatives, based on the 16S rRNA gene. Sequences generated in this study are from clone libraries ("clone"), bacterial cultivation ("isolate"), and amplicon sequencing ("barcode"). A neighbor-joining tree with Jukes-Cantor two-parameter distances is shown. None of the nodes are supported by bootstraps, reinforcing the very close relationship among known *Vibrio* species, and the need for a more specific genomic taxonomy framework. Genbank accession numbers are indicated in parentheses. *Vibrio* barcodes shown in Figures 3 are highlighted in bold text. At right are scanning electron microscopy or blood agar hydrolysis images of bacterial isolates corresponding to the main *Vibrio* groups recovered from blood feeding marine crustaceans and leeches.

SUPPLEMENTARY FIGURE S2
Phylogenetic relationships of bacteria associated with blood feeding marine crustaceans and leeches (based on 16S rRNA gene amplicon sequencing, designated by names beginning with 'OBF'), compared to select close relatives recovered during other investigations. Genbank accession numbers are indicated in parentheses.
(A) gammaproteobacteria, including unclassified members and Porticoccaceae + Oceanospirillaceae, **(B)** alphaproteobacteria, including unclassified members and Rhodobacteraceae **(C)** betaproteobacteria, including Rhodocyclaceae, and **(D)** Flavobacteriacea. For each, a neighbor-joining tree with Jukes-Cantor two-parameter distances is shown. The numbers at the nodes represent maximum parsimony bootstrap values from 1000 replicate samplings (only values >60% are shown).

SUPPLEMENTARY FIGURE S3
Comparisons of the microbiome diversity of adult obligate blood feeding crustaceans and leeches, versus egg and cocoons, respectively. 16S rRNA gene sequence similarity was analyzed using a non-metric multidimensional scaling (NMDS) ordination plot based on Bray-Curtis similarity resemblance. The tables below compare the Shannon diversity index in both the adults and eggs (or cocoons), as well as the relative abundance of select dominant bacterial groups.

SUPPLEMENTARY FIGURE S4
Fluorescent visualization using a nonsense probe, labeled with a Cy3 fluorescent molecule, counterstained with DAPI. **(A)** The bony fish leech *Pterobdella occidentalis*, showing a negative signal compared to the positive signal shown in Figures 7H. Scale bar 200 μm. **(B)** The parasitic isopod *Elthusa vulgaris*, showing a negative signal compared to the positive signal shown in Figures 7F. Scale bar 100 μm. **(C)** *Lernanthropus latis,* showing a negative signal, compared to panel E. Scale bar 20 μm. **(D)** The elasmobranch leech *Branchellion lobata*, showing a negative signal, compared to panel F. Scale bar 20 μm. **(E)** *Lernanthropus latis,* showing the result from the *Vibrio*-specific fluorescent probe revealing rod-shaped bacterial cells, shown in orange via Cy3. Scale bar 20 μm. (F) *Branchellion lobata,* showing the result from the *Vibrio*-specific fluorescent probe revealing rod-shaped bacterial cells, shown in orange via Cy3. Scale bar 20 μm.

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 21 June 2023
DOI 10.3389/fmicb.2023.1188155

OPEN ACCESS

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RECEIVED 17 March 2023
ACCEPTED 31 May 2023
PUBLISHED 21 June 2023

CITATION
Dong L, Li Y, Yang C, Gong J, Zhu W, Huang Y, Kong M, Zhao L, Wang F, Lu S, Pu J and Yang J (2023) Species-level microbiota of ticks and fleas from *Marmota himalayana* in the Qinghai-Tibet Plateau.
Front. Microbiol. 14:1188155.
doi: 10.3389/fmicb.2023.1188155

Species-level microbiota of ticks and fleas from *Marmota himalayana* in the Qinghai-Tibet Plateau

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Introduction: Ticks and fleas, as blood-sucking arthropods, carry and transmit various zoonotic diseases. In the natural plague foci of China, monitoring of *Yersinia pestis* has been continuously conducted in *Marmota himalayana* and other host animals, whereas other pathogens carried by vectors are rarely concerned in the Qinghai-Tibet Plateau.

Methods: In this study, we investigated the microbiota of ticks and fleas sampling from *M. himalayana* in the *Qinghai-Tibet* Plateau, China by metataxonomics combined with metagenomic methods.

Results: By metataxonomic approach based on full-length 16S rDNA amplicon sequencing and operational phylogenetic unit (OPU) analyses, we described the microbiota community of ticks and fleas at the species level, annotated 1,250 OPUs in ticks, including 556 known species and 492 potentially new species, accounting for 48.50% and 41.71% of the total reads in ticks, respectively. A total of 689 OPUs were detected in fleas, consisting of 277 known species (40.62% of the total reads in fleas) and 294 potentially new species (56.88%). At the dominant species categories, we detected the *Anaplasma phagocytophilum* (OPU 421) and potentially pathogenic new species of *Wolbachia, Ehrlichia, Rickettsia*, and *Bartonella*. Using shotgun sequencing, we obtained 10 metagenomic assembled genomes (MAGs) from vector samples, including a known species (*Providencia heimbachae* DFT2), and six new species affliated to four known genera, i.e., *Wolbachia, Mumia, Bartonella*, and *Anaplasma*. By the phylogenetic analyses based on full-length 16S rRNA genes and core genes, we identified that ticks harbored pathogenic *A. phagocytophilum*. Moreover, these potentially pathogenic novel species were more closely related to *Ehrlichia muris, Ehrlichia muris* subsp. *eauclairensis, Bartonella rochalimae*, and *Rickettsia limoniae*, respectively. The OPU 422 Ehrlichia sp1 was most related to *Ehrlichia muris* and *Ehrlichia muris subsp. eauclairensis*. The OPU 230 *Bartonella* sp1 and *Bartonella* spp. (DTF8 and DTF9) was clustered with *Bartonella rochalimae*. The OPU 427 *Rickettsia* sp1 was clustered with *Rickettsia limoniae*.

Discussion: The findings of the study have advanced our understanding of the potential pathogen groups of vectors in marmot (*Marmota himalayana*) in the Qinghai-Tibet Plateau.

KEYWORDS

tick, flea, *Marmota himalayana*, *Anaplasma phagocytophilum*, *Wolbachia*, *Bartonella*

Introduction

Vector-borne diseases (VBDs) are infections that are primarily transmitted through an invertebrate, generally, insects. With the global climatic alteration and human activity amplification, the VBDs pose a significant burden on public health and economic development. It is estimated that 80% of the population in the world is at risk of two or more VBDs. They account for 17% of all infectious diseases and cause more than 700,000 deaths annually (Golding et al., 2015; WHO, 2017; Chala and Hamde, 2021). As the largest developing country in the world, China has made great progress in the prevention and treatment of infectious diseases. However, emerging infectious diseases are a new challenge for China (Wang et al., 2008).

Ticks are obligate blood-sucking arthropods that can parasitize vertebrates and transmit a wide variety of infectious microorganisms such as viruses, bacteria, spirochetes, protozoans, and parasites (Parola, 2004). As a major contributor to vector-borne diseases, a huge number of novel tick-borne disease agents have been reported such as borreliosis, ehrlichioses, anaplasmosis, and tick-borne rickettsial diseases. Among the 109 tick-borne pathogens identified in China (Zhao et al., 2021) between 1950 and 2018, 65 were newly identified. Plenty of human cases have been confirmed for tick-borne infections with *Borrelia*, *Anaplasmataceae*, *Babesia*, and spotted fever group *Rickettsiae* (Fang et al., 2015). Fleas are blood-sucking arthropods that parasitize mammals and birds. They are known for transmitting flea-borne *Yersinia pestis* causing plague (Zeppelini et al., 2016). In recent years, increasing numbers of flea-borne diseases, including murine typhus, epidemic typhus, and flea-borne spotted fever have been reported across the world (Bitam et al., 2010). Fleas have also been reported to transmit *Bartonella*, including *Bartonella henselae*, the agent cat-scratch disease (Chomel et al., 2006; Gutiérrez et al., 2015).

Marmots (*Marmota himalayana*) were identified as the predominant host of *Yersinia pestis* in the Qinghai-Tibet Plateau, China (Ben-Ari et al., 2012). In the natural plague foci of China, such as Qinghai and Xinjiang, marmots and other animal hosts have been continuously monitored under China's Plague Surveillance Program (Qin et al., 2022). Our previous studies have found the possibility of marmots to be hosts and reservoirs for pathogens including tick-born encephalitis virus (Dai et al., 2018) and delta-Coronaviruses (Zhu et al., 2021). In recent years, there have been some studies on vector-borne pathogens in this area, such as the research of spotted fever group *Rickettsia* infecting ticks, yaks, and Tibetan sheep (He et al., 2021b) and the molecular detection of *Anaplasm*, *Babesia*, and *Theileria* in yaks and Tibetan sheep (He et al., 2021a). However, the characterization of species-level microbiota of vectors, especially in fleas, is lacking in marmots. In the present study, we described the microbiota community of ticks and fleas sampling from marmots in Qinghai-Tibet Plateau, China. We aimed to discover the profile of tick-borne and flea-borne pathogens or potentially new pathogenic bacterial species, providing etiological information for vector infectious disease prevention and control.

Materials and methods

Samples collection

The ticks and fleas were collected from *M. himalayana* in Tongren, Huangnan Tibetan Autonomous Prefecture (Altitude: 3273 m; N: 35°49′, E: 102°28′), Qinghai Province, China, from July to September 2018. Marmots were collected for routine surveillance and screened for plague in Autumn 2018. All the marmots were captured and preliminarily detected *Y. pestis* F1 antibody by the colloidal gold method. Then, ticks and fleas parasitized on the surface of marmots were collected and stored on the principle of one marmot per frozen tube. All the vectors were transported to the local lab, and subsequently, ticks and fleas were identified as adults by professionals using a DM-500 binocular biological microscope, referring to the reference book of Cai et al. (1997). All the samples were transported to our laboratory in Beijing and stored at −80°C prior to DNA extraction. The sampling work was conducted by Qinghai Institute for Endemic Disease Prevention and Control for seasonal vector surveillance. The ethical practice was approved by the Ethical Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, China (No. ICDC-2018004).

DNA extraction and full-length 16S rDNA amplification sequencing

A total of 25 ticks and 24 fleas were enrolled in our experiment, which were divided into eight pools for each sample ticks and fleas according to the engorgement level (Supplementary Table S1). Ticks and fleas were surface-sterilized in 10% bleach and then washed twice in 99% ethanol to remove bacteria from the external body. Sixteen vector samples and two blank control groups were homogenized (Liu et al., 2017; Adegoke et al., 2020) separately in the PBS solution by the TissueLyser II (Qiagen, Germany) at 24 Hz, 37°C for 2 min, three times. The TissueLyser II system was achieved through high-speed shaking with beads, which beat and grid samples, and also simultaneously homogenized samples to facilitate subsequent purification procedures. The total DNA was then extracted from the supernatant using the QIAamp DNA Mini Kit (Qiagen, Germany). The quality of DNA extraction was checked by NanoDrop 2000 (Thermo Fisher Scientific, USA). The almost full-length 16S rRNA gene was amplified using the universal primers 27F/1492R with 16 nt symmetric (reverse complement) barcodes tagged at the 5′ end, as described previously (Yang et al., 2020). The libraries of PCR products were generated, followed by sequencing on the PacBio Sequel platform at Tianjin Biochip Corporation, China.

The raw sequences generated were filtered and cleaned using the previous pipeline described by Meng et al. (2017) and Yang et al. (2020), i.e., the raw sequencing data were initially processed based on the PacBio SMRT Link (version 6.0.0) pipeline, QIIME (Bolyen et al., 2019) was used to split data and filter out adapters, primers, ambiguous bases, and chimeras were detected and removed using the UCHIME (Edgar et al., 2011) algorithm implemented in

USEARCH (version 11.0.667; option: -uchime_ref -strand plus -nonchimeras) with the RDP Gold reference database.

Species-level taxonomy by operational phylogenetic unit analyses

High-quality sequences of the full-length 16S rRNA gene were clustered into operational taxonomic units (OTUs) with an identity threshold of 98.7% by the USEARCH pipeline. Then, all the representative sequences for each OTU were grouped into operational phylogenetic units (OPUs) using the tool Arb by the visual inspection of the final phylogenetic trees. The reference database used was LTP132 (which was the latest version at the time of this study). The detailed analysis process and classification criteria of OPU were shown in our previous studies (Meng et al., 2017; Bai et al., 2018; Yang et al., 2020). In brief, each OPU consisting of one or more OTUs can be equated to a species-level taxon. When an identity value of OTU representatives with a type strain was >98.7%, it was identified as known species. A putative new species within a specific genus was defined when the identity values of OTU representatives with the closest type strain were <98.7 and >94.5%. When OTUs represent a sequence whose membership position was not clear but was closely affiliated to a known genus, family, or higher taxa, it was listed as a potential new "higher taxa." Taxa around the OTU sequences that were represented by a large number of uncultured organisms and were not attached to any of the internal taxa were referred to as "uncultured taxa" categories.

Shotgun metagenomic sequencing, assembly, and contig binning

The DNA material used for shotgun metagenomic sequencing was the same as that described in 16S rRNA-based amplicon sequencing section. DNA degradation, contamination, and concentration were monitored by agarose gel electrophoresis and Qubit® 2.0 Fluorometer. Then, DNA libraries were constructed, purified, analyzed, quantified, and sequenced on the Illumina PE150 platform (150-bp, paired-end) at the Novogene Co., Ltd. (Beijing, China).

The raw paired-end reads were quality-filtered to remove the adapter and low-quality sequences using the Readfq (V8, https://github.com/cjfields/readfq). Then, the reads mapped to the host genomic DNA by Bowtie2 (version 2.2.4, http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) with the following parameter settings: –end-to-end, –sensitive, -I 200, and -X 400 were filtered out. The sequence data were assembled individually by MEGAHIT (version 1.0.4, https://github.com/voutcn/megahit) using the parameter settings: –presets meta-large (–end-to-end, –sensitive, -I 200, -X 400). Next, the assembled contigs were binned with three different tools using the default settings in MetaWRAP (Uritskiy et al., 2018) (version 1.3.2) pipeline, and the completeness and contamination of the reassembled metagenomic assembled genomes (MAGs) were estimated by CheckM (Parks et al., 2015) (version 1.0.13). The reassembled MAGs were classified using the Genome Taxonomy Database Toolkit (GTDB-Tk version 1.3.0) based on the GTDB RefSeq_95 database. The taxonomic assignment was defined by a combination of the placement of the query genome in the GTDB reference tree, the RED of the query genome, and Average Nucleotide Identity (ANI) (Ciufo et al., 2018; Jain et al., 2018) to reference genomes.

Phylogenetic analyses of the potential vector-borne pathogens

The phylogenetic analyses of full-length 16S rRNA gene amplicon sequencing were performed with MEGA X (https://www.megasoftware.net/) using the maximum-likelihood (ML) algorithm. Multiple alignments were performed with the program CLUSTAL_W, and all cases were evaluated based on 1,000 bootstrap replicates. The phylogenetic analyses of reassembled MAGs were performed with FastTree (Price et al., 2009) by using the neighbor-joining (NJ) algorithm, which was generated based on core genes that were extracted from the reference whole genomes and visualized in Dendroscope 3.0.

Results

Species-level microbiota of ticks and fleas

A total of 16 vector pools were sequenced, with eight pools each for ticks and fleas (Supplementary Table S1). The PacBio sequel sequencing platform rendered a total of 135,040 and 98,090 raw reads for ticks and fleas, respectively. After quality filtering and chimera removal, a total of 110,760 (80.50%) and 70,502 (71.10%) high-quality reads were obtained from ticks and fleas, with an average of 13,845.00 ± 6,588.00 per tick pool and 8,812.75 ± 2,681.68 per flea pool. The average length was 1,437.00 ± 15.07 and 1,453.63 ± 7.02 nucleotide (nt) per read for ticks and fleas, respectively (Supplementary Table S2). The near full-length 16S rRNA gene sequences were clustered into 4,789 and 2,170 OTUs in respective ticks and fleas at an identity threshold of 98.7% using the USEARCH pipeline. Subsequently, a total of 1,250 and 689 operational phylogenetic units (OPUs) were obtained for ticks and fleas, respectively, with an average of 303.25 ± 164.84 per tick pool and 153.36 ± 96.37 per flea pool (Supplementary Table S3). We further classified these OPUs into four categories: (i) known species (556 OPUs, relative abundance 48.50% vs. 277 OPUs, 40.62% in ticks and fleas, respectively); (ii) potentially new species (492 OPUs, 41.71% vs. 294 OPUs, 56.88%); (iii) higher taxa (133 OPUs, 6.19% vs. 83 OPUs, 2.02%); and (iv) uncultured bacteria (69 OPUs, 3.60% vs. 35 OPUs, 0.48%; Supplementary Table S3 and Supplementary Figure S1). It can be noted that the vast majority of the flora was identified at the species level (known species and potentially new species), indicating the relative abundance of dominant taxa reached 90.21% and 97.50% in ticks and fleas, respectively.

The potential vector-borne pathogens detected in ticks and fleas at the species level

In ticks, the 556 known species and 492 putative new species affiliated to 19 phyla, 49 classes, 96 orders, 177 families, and 431 genera, accounting for 90.21% of the total reads (Figure 1A and Supplementary Figure S1). The top 10 most abundant species in ticks represented 74.76% of the total reads, ranging from 27.25% to 97.38% in individual tick pools (Figure 1C). Of these 10 species, the *Anaplasma phagocytophilum* (OPU 421) and *Ehrlichia* sp1 (OPU 422) were presented, and their relative abundance were 5.11% and 3.37%, respectively, and which were indicated by red arrows and words in Figures 1A, C. However, *Bartonella* sp1 (OPU 230) had a relatively lower abundance of 0.01%. In flea samples, most of the reads (97.50%) were annotated to the species level, including 277 known species and 294 putative new species (Figure 1B and Supplementary Figure S1). The top 10 species accounted for 90.51% of the total reads, ranging from 84.51% to 96.07% in individual flea pools (Figure 1D). The potential novel species affiliated to vector-borne pathogenic genera including *Rickettsia* and *Bartonella* were founded in these top 10 species, and their relative abundance were 3.01% and 0.75%, respectively.

In addition, *Wolbachia* were detected in the top 10 abundance at the species level in both ticks and fleas. The relative abundance of *Wolbachia* sp1 (OPU 416) were 4.67% in ticks and 38.47% in fleas.

Notably, the known vector-borne pathogen *A. phagocytophilum* and potentially novel species affiliated with vector-borne pathogenic genera, including *Ehrlichia*, *Rickettsia*, and *Bartonella* were detected in the dominant species categories of the microbiota community in ticks and fleas, accounting for 13.16% and 42.23% of the total reads in ticks and fleas, respectively (Figure 2). Hence, we intend to acquire the complete genomes of these potential pathogens for deep analysis.

The metagenomic assembled genomes of ticks and fleas

We used the same DNA samples as full-length 16S rDNA sequencing for shotgun metagenomic sequencing. An average of 7,040.24 ± 747.37 and 7,396.88 ± 627.33 Mb high-quality reads data (paired-end, 150-bp) per sample pools were generated in ticks and fleas, respectively. The *de novo* assembly produced a mean of 474,197 ± 48,301 and 287,426 ± 84,212 kb contigs per sample pool comprising respective ticks and fleas (Supplementary Table S4). Finally, we recovered nine MAGs (bins) from the assembled contigs in fleas and one MAG in ticks. Detailed descriptions of the 10 MAGs and their proposed nomenclatures were provided in Supplementary Table S5. A total of six out of 10 MAGs had >90% completeness and <5% contamination, while three MAGs showed >70% completeness and <10% contamination, and one MAG had <50% completeness and <15% contamination (Supplementary Table S5). Moreover, 10 MAGs (>1 Mbp) were subsequently identified using the Genome Taxonomy Database Toolkit (GTDB-Tk) combined with ANI value calculation. It was shown that one MAG (f5_bin2) belonged to known bacterial species (*Providencia heimbachae* DFT2; >95% ANI with a complete genome), six MAGs could be assigned to undescribed species affiliated to four known genera, including *Wolbachia*, *Mumia*, *Bartonella*, and *Anaplasma*, and the remaining three MAGs had low ANI values (<85%), demonstrating that they may belong to the family *Rickettsiaceae* (f5_bin1, f6_bin6) and order *Acidimicrobiales* (f6_bin5).

Phylogenetic analyses of the potential vector-borne pathogens

We identified the known vector-borne pathogen *A. phagocytophilum* and potentially new species affiliated with vector-borne pathogenic genera, including *Ehrlichia*, *Rickettsia*, and *Bartonella*, using full-length 16S rRNA gene sequencing. Further through metagenomic data, we recovered the MAGs of *Bartonella* and *Anaplasma* from fleas and ticks. To further confirm the taxonomic positions of these potential vector-borne pathogens, we constructed the phylogenetic trees based on the full-length 16S rRNA genes and core genes using the ML and NJ algorithms, respectively. The OPU 421 identified in ticks with 5,431 reads affiliated as an independent cluster with *A. phagocytophilum* (U02521, NR 044762, and AY537213) with an average 16S rRNA gene sequence similarity of 99.72%, possibly representing a known species *A. phagocytophilum* (Figure 3A). The phylogenomic tree based on 208 core genes showed that *Anaplasma* sp. DTF10 (tick_bin1) formed a branch with *A. phagocytophilum* (NC 021880; Figure 3B). The ANI value between *Anaplasma* sp. DTF10 and *A. phagocytophilum* NC 021880 was 89.3%, indicating that it could be assigned as a new species of *Anaplasma*. Phylogenetic analysis based on the 16S rRNA gene sequences revealed that OPU 422 (3,586 reads in ticks) was related to *Ehrlichia muris* (HM543745, NRO25962, NR121714, and U15527) and *Ehrlichia muris* subsp. *eauclairensis* (HM543745; Figure 5A) with a maximum 16S rRNA gene sequence similarity of 98.4%. Thus, OPU 422 could represent a new species belonging to the genus *Ehrlichia*.

The OPU 230 (469 reads in fleas) were placed in a clade with *Bartonella rochalimae* (DQ683196) based on the phylogenetic analyses of the 16S rRNA gene (Figure 4A). The representative sequences of OPU 230 *Bartonella* sp1 showed an average identity of 98.40 ± 0.01% with *B. rochalimae* (DQ683196). A phylogenomic tree based on 333 core genes showed that the two MAGs (f6_bin8, f8_bin9) of *Bartonella* spp. (DTF8 and DTF9) formed a distinct clade and were clustered with *B. rochalimae* (NZ KL407337), which was consistent with the phylogenetic tree of 16S rRNA gene sequences (Figure 4B). The ANI values between *Bartonella* spp. (DTF8 and DTF9) and *B. rochalimae* (NZ KL407337) ranged from 91.79% to 94.52 %, indicating two uncultured new species within the genus *Bartonella*. The representative sequences of OPU 427 *Rickettsia* sp1 (1,897 reads in fleas) were shown to be clustered with *Rickettsia limoniae* (AF322442; Figure 5B), with an average identity of 97.23 ± 0.025 %, indicating that OPU 427 could be a new species affiliated to genus *Rickettsia*.

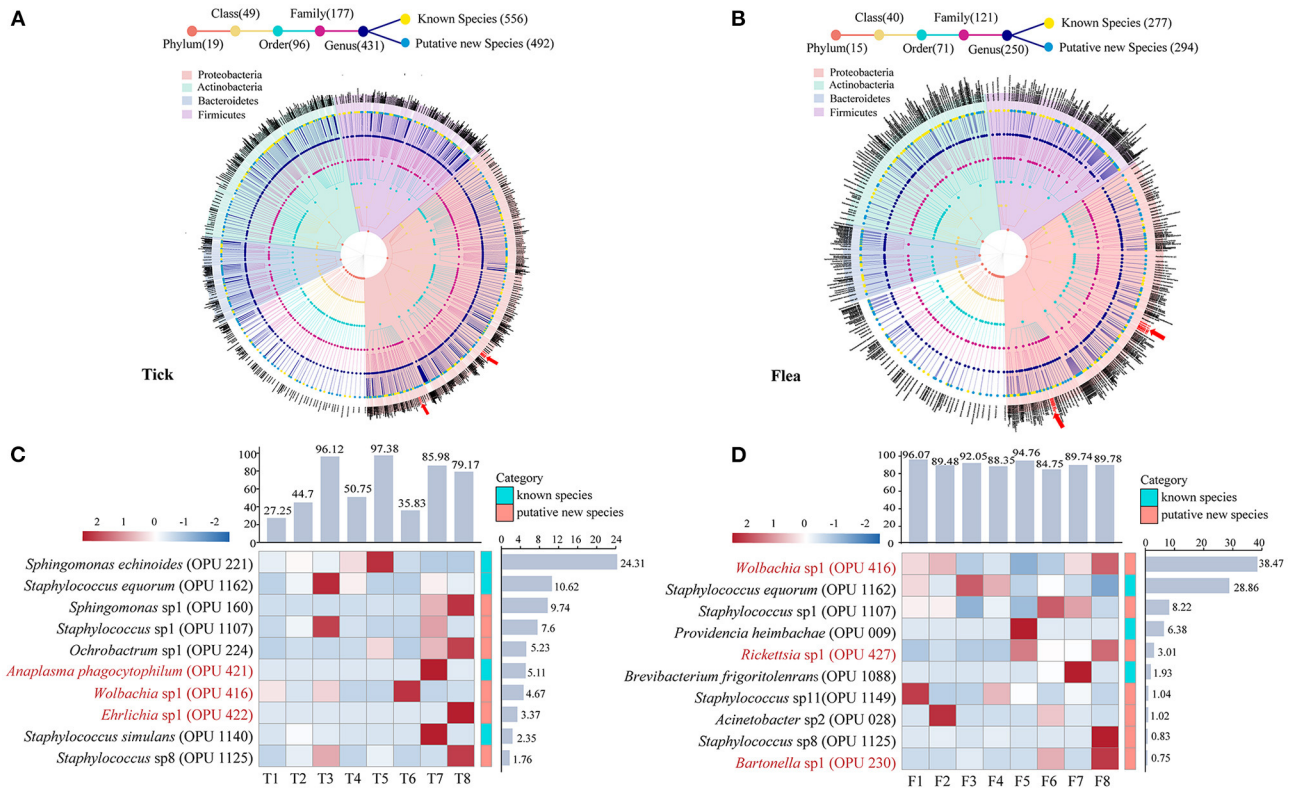

FIGURE 1
Taxonomic profile and dominant taxa in ticks and fleas at species level. Taxonomic trees of **(A)** 556 known species and 492 putative new species in ticks and **(B)** 277 known species and 294 putative new species in fleas. The heat map and relative abundance of the top ten abundant bacteria at the species level **(C)** in ticks and **(D)** in fleas. Each dot represents a taxonomic entity. From the inner to the outer circles, the taxonomic levels range from phylum to species. Dots of different colors indicate different taxonomic levels according to the color key shown. The total number at different hierarchical levels is displayed in brackets. The potential pathogens are indicated by red arrows and words in the figure. Different colored squares represent different phyla according to the color key shown.

Discussion

In the present study, we have described the microbiota community in ticks and fleas collected from *M. himalayana* in Qinghai Tibet Plateau, China. By using the metataxonomic analyses combined with shotgun metagenomics, we obtained species-level microbiota profiles of ticks and fleas, with 90.21% and 97.50% of the total reads annotated at the species level (known species and potentially new species), respectively. The metataxonomic method based on the full-length 16S rRNA gene amplicon sequencing had been applied in the investigation of the microbiota in vultures, Tibet antelopes, and human gut microbiota (Meng et al., 2017; Bai et al., 2018; Yang et al., 2020) in our previous studies. We identified a total of 1,250 and 689 OPUs (species-level taxon) in ticks and fleas, respectively. The major bacterial at phylum-level microbiota of ticks and fleas in our study included *Proteobacteria* and *Firmicutes*, followed by *Cyanobacteria* and *Actinobacteria*, which was consistent with the previous studies of fleas (Supplementary Figure 2; Hawlena et al., 2013), ticks (Andreotti et al., 2011; Thapa et al., 2019), and other vectors (Minard et al., 2013; Yun et al., 2014).

Notably, at the species level, we detected the known vector-borne pathogen *A. phagocytophilum* (OPU 421) and several novel species affiliated with vector-borne pathogenic genera, *Ehrlichia*, *Bartonella*, and *Rickettsia*, in the present study and performed deep analysis combined with metagenomic assembly and binning. The *A. phagocytophilum*, known as the causative agent of anaplasmosis, is responsible for approximately 6,000 cases of human granulocytic anaplasmosis per year in the USA (Bakken and Dumler, 2015). Previous studies have reported that the *A. phagocytophilum* has been divided into pathogenic *A. phagocytophilum* and the closely related nonpathogenic *A. phagocytophilum*-like (APL) species (Ben Said et al., 2017; Seo et al., 2018; Yan et al., 2022). From the phylogenetic analyses based on 16S rRNA gene sequences, the OPU 421 identified ticks in this study clustered with pathogenic *A. phagocytophilum* (Figure 3A), indicating that the ticks in the Qinghai-Tibet Plateau harbored pathogenic *A. phagocytophilum*. Recently, Duan et al. (2022) in their study detected the *A. phagocytophilum* for the first time in *M. himalayana* in Tibet, China, confirming that *M. himalayana* is a reservoir for *A. phagocytophilum*. Most importantly, they have found that plague may be exacerbated in the presence of *A. phagocytophilum* and humans that are at risk of infection by exposure to such marmots through ticks and fleas, potentially leading to a complicated disease.

In addition, using the phylogenetic analyses based on full-length 16S rRNA genes and core genes, we found that these

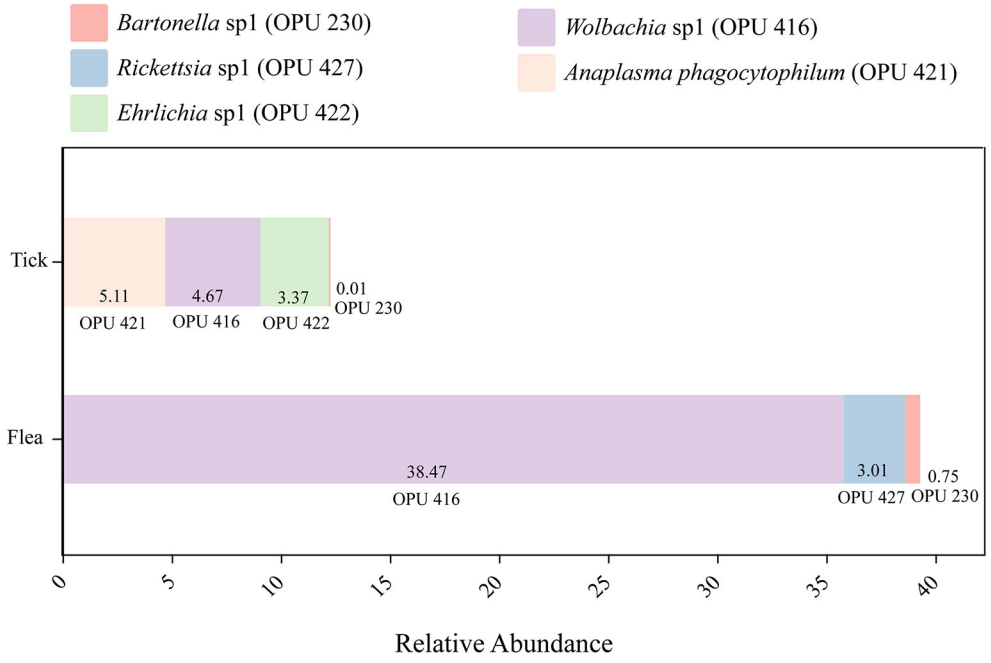

FIGURE 2
The relative abundance of the potential pathogens in ticks and fleas including *Anaplasma phagocytophilum* (OPU 421), *Ehrlichia* sp1 (OPU 422), *Bartonella* sp1 (OPU 230), *Rickettsia* sp1 (OPU 427), and *Wolbachia* sp1 (OPU 416).

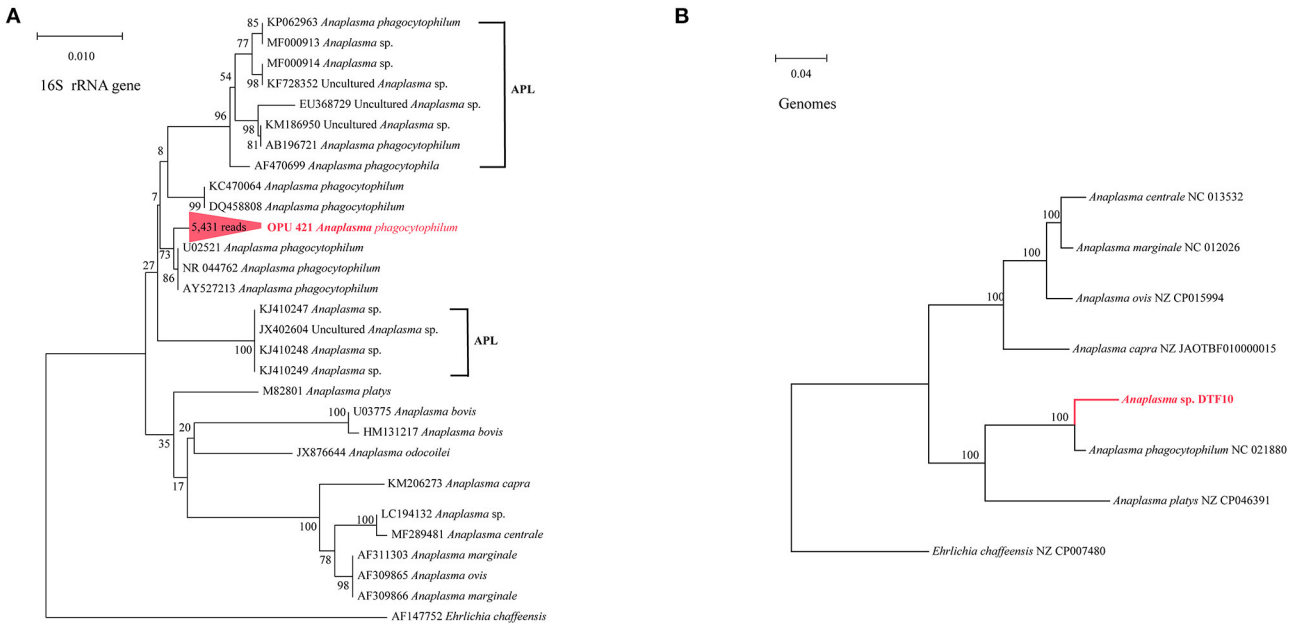

FIGURE 3
Phylogenetic trees based on full-length 16S rRNA gene sequences and genomic core genes revealed the positions of **(A)** the OPU 421 *Anaplasma phagocytophilum* **(B)** the *Anaplasma* sp. DTF10 (208 core genes). The phylogenetic trees were constructed using maximum-likelihood algorithms based on full-length 16S rRNA gene sequences and neighbor-joining (NJ) algorithms based on core genes. The APL represents genetically related *A. phagocytophilum*-like (APL) species. Trees showed the precise position of the OPUs and genomes of the identified species compared with members of the most closely related reference sequences. Red labels are the OPUs and genomes of the identified species, while known species are shown in black.

novel species were most close to *E. muris*, *E. muris* subsp. *eauclairensis*, *B. rochalimae*, and *R. limoniae*, respectively. *E. muris* was originally isolated in 1983 and was identified as the cause of the patients in Minnesota and Wisconsin in 2009 (Pritt et al., 2011). In addition, the *E. muris* subsp. *eauclairensis* was recognized as the etiological agent of human ehrlichiosis

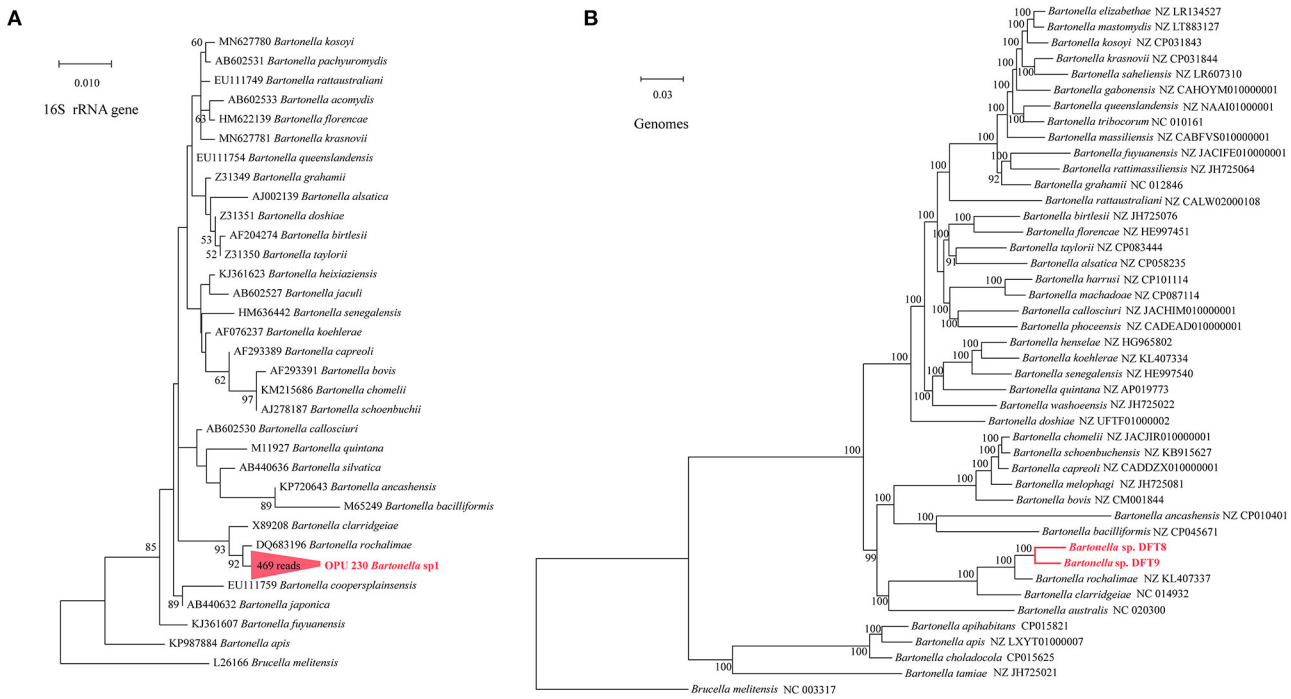

FIGURE 4
Phylogenetic trees based on full-length 16S rRNA gene sequences and genomic core genes revealed the positions of **(A)** the OPU 230 *Bartonella* sp1 and **(B)** the *Bartonella* sp. DTF8 and the *Bartonella* sp. DTF9 (333 core genes). The phylogenetic trees were constructed using the maximum-likelihood and neighbor-joining (NJ) algorithms based on full-length 16S rRNA gene sequences and core genes. Trees showed the precise position of the OPUs and genomes of the identified species compared with members of the most closely related reference sequences. Red labels are the OPUs and genomes of the identified species, while known species are shown in black.

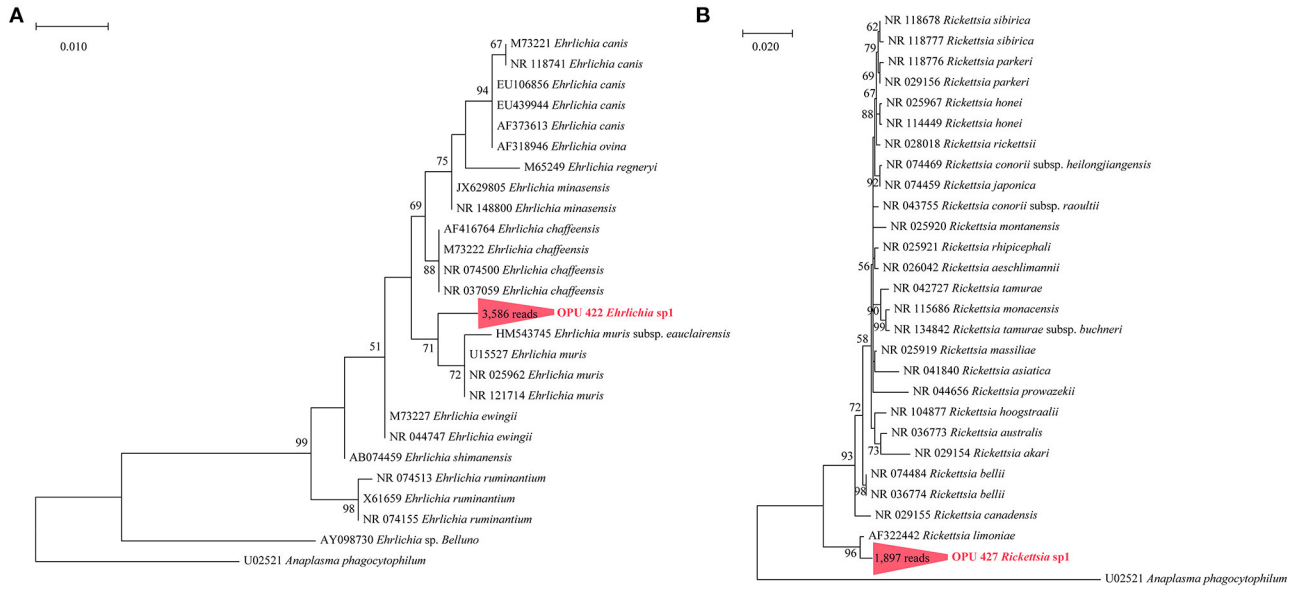

FIGURE 5
Phylogenetic trees based on full-length 16S rRNA gene sequences revealed the positions of **(A)** the OPU 422 *Ehrlichia* sp1 and **(B)** the OPU 427 *Rickettsia* sp1. The phylogenetic trees was constructed using a maximum-likelihood algorithm based on full-length 16S rRNA gene sequences. Trees showed the precise position of the OPUs of the identified species compared with members of the most closely related reference sequences. Red labels are the OPUs of the identified species, while known species are shown in black.

in Minnesota and Wisconsin (Lynn et al., 2019). Although transmission to human appears to be limited to Minnesota and Wisconsin, this species could be a potential hazard to humans in the studied area. *Bartonella* were the most frequently identified bacteria in the fleas collected from rodents in southern Indiana, USA (Hawlena et al., 2013). *Bartonella rochalimae* can

cause intra-erythrocytic infections in mammals and can cause disease manifestations that include bacteremia, splenomegaly, fever, and myalgia in humans. This pathogen has been identified in *Xenopsylla cheopis* fleas collected from *Rattus norvegicus* rats in Los Angeles, California (Billeter et al., 2011). *Rickettsia* can cause a group of diseases such as spotted fever group rickettsioses. However, with more *Rickettsia* species identified, plenty of *Rickettsia* were classified into endosymbionts rather a pathogen. *Rickettsia limoniae* has been reported from the microbiome of non-agricultural insects including the cranefly *Limonia chorea* (Perlman et al., 2006). However, its pathogenicity is unclear to date. In conclusion, these undescribed novel species, whether they can lead to diseases, need further study and surveillance.

Another important observation from our study was the detection of the vertically transmitted *Wolbachia* bacteria among these ticks and fleas in the present study. *Wolbachia* are obligate endosymbionts of bacteria that have been identified in various arthropods, including ticks, fleas, and mosquitos (Plantard et al., 2012; Duan et al., 2020; Flatau et al., 2021). Previous studies have reported that *Wolbachia* are essential for the survival and reproduction of the host and are involved in molting, embryogenesis, growth, and survival of the host. It can profoundly influence the ecology and evolution of its host through a wide range of symbiotic interactions. Currently, *Wolbachia* are the staple means of controlling vector-borne diseases due to their unique ability to spread through insect populations and to affect vector competence through pathogen protection. However, our understanding of their biology is still in its infancy and needs further study (Landmann, 2019; Manoj et al., 2021).

There are some shortcomings in this study. Although we obtained several metagenomic assembled genomes from ticks and fleas, we did not isolate potentially pathogenic species successfully. By controlling the density of marmots, the plague was rarely reported in the Qinghai-Tibet Platea in recent years. Thus, vector sample collection becomes more difficult, because it is only possible to collect ticks and fleas after a live marmot has been captured. This leads to another limitation in this study, which is a small sample size of ticks and fleas that we collected.

Conclusion

By using the metataxonomic analysis based on full-length 16S rRNA gene amplicon sequencing, we comprehensively characterized the microbiota community in ticks and fleas collected from *M. himalayana* found in the Qinghai-Tibet Plateau at the species level for the first time. Notably, we identified the pathogenic *A. phagocytophilum* and potentially new vector-borne pathogens affiliated with genera *Ehrlichia*, *Bartonella*, and *Rickettsia*, and we performed deep analysis combined with metagenomic assembly and binning. The findings of the study have advanced our understanding of the potential pathogen groups of vectors in marmots in the Qinghai-Tibet Plateau, and we hope to provide etiological information for vector infectious disease prevention and control in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA898891.

Ethics statement

The animal study was reviewed and approved by Ethical Committee of National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, China (No. ICDC-2018004).

Author contributions

Conceptualization: LD and JY. Methodology: LD, JP, and JY. Data curation: JP and JY. Formal analysis: LD, JP, and CY. Visualization: LD, YL, CY, and JG. Writing—original draft: LD. Writing—review and editing: JY, JP, YL, WZ, YH, MK, LZ, and FW. All authors contributed to the article and approved the final version of the manuscript.

Funding

This work was supported by grants from the National Key R&D Program of China (2019YFC1200501) and Research Units of Discovery of Unknown Bacteria and Function (2018RU010).

Acknowledgments

We would like to appreciate Professor Hongjian Chen and his team for providing help on vector sampling and identification. We also thank Professor Yaming Sun and his team for the technical assistance with PacBio sequencing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1188155/full#supplementary-material

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