The microbiota changes of the brown dog tick, *Rhipicephalus sanguineus* under starvation stress

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*Rhipicephalus sanguineus*, the brown dog tick, is the most widespread tick in the world and a predominant vector of multiple pathogens affecting wild and domestic animals. There is an increasing interest in understanding the role of tick microbiome in pathogen acquisition and transmission as well as in environment–vector interfaces. Several studies suggested that the tick microbial communities are under the influence of several factors including the tick species, dietary bloodmeal, and physiological stress. Compared with insects, very little of the microbial community is known to contribute to the nutrition of the host. Therefore, it is of significance to elucidate the regulation of the microbial community of *Rh. Sanguineus* under starvation stress. Starvation stress was induced in wild-type adults (1 month, 2 months, 4 months, 6 months) and the microbial composition and diversity were analyzed before and after blood feeding. After the evaluation, it was found that the microbial community composition of *Rh. sanguineus* changed significantly with starvation stress. The dominant symbiotic bacteria *Coxiella* spp. of *Rh. sanguineus* gradually decreased with the prolongation of starvation stress. We also demonstrated that the starvation tolerance of *Rh. sanguineus* was as long as 6 months. Next, *Coxiella*-like endosymbionts were quantitatively analyzed by fluorescence quantitative PCR. We found a pronounced tissue tropism in the Malpighian tubule and female gonad, and less in the midgut and salivary gland organs. Finally, the blood-fed nymphs were injected with ofloxacin within 24 h. The nymphs were allowed to develop into adults. It was found that the adult blood-sucking rate, adult weight after blood meal, fecundity (egg hatching rate), and feeding period of the newly hatched larvae were all affected to varying degrees, indicating that the removal of most symbiotic bacteria had an irreversible effect on it.

**KEYWORDS**

*Rhipicephalus sanguineus*, starvation stress, coxiella, tick, microbiota community, symbiotic bacteria
1 Introduction

Food is an essential source of nutrition for arthropods and an important external factor for survival. A long-term lack of food will affect the growth and reproduction of arthropods and may even lead to death. Symbionts in arthropods make essential contributions to nutrient supply. Such nutritional symbioses are crucial in many obligate blood-feeders, such as ticks, tsetse flies and bed bugs, where bacterial symbionts synthesize B vitamins lacking in the blood meal (Nikoh et al., 2014; Ben-Yosef et al., 2020). Bacterial cells provide related nutrients necessary for survival and reproduction, revealing an evolutionary pathway to obligate trophic symbionts in which host and symbionts merge into a coherent organism and highlighting the parasitic - mutualistic evolutionary continuum (Hosokawa et al., 2010; Lockwood et al., 2016). Similar correlational studies have been incomplete in ticks. The maternally inherited symbionts in the African soft tick Ornithodoros moubata provide missing B vitamins; these nutritional symbionts are critical for tick growth and survival in adulthood (Duron et al., 2018). Identifying and characterizing the tick microbiota is thus crucial to better understand tick-microbe interactions. While the microbiota of several tick species have been studied, little information is currently available on the microbiota of Rhipicephalus sanguineus which is the most widespread tick in the world. R. sanguineus is distributed in tropical and subtropical and temperate regions, and it breeds in human habitats (Dantas-Torres, 2010). They carry a variety of protozoa and microorganisms, including Ehrlichia canis, Rickettsia, Mycoplasma, Babesia microti, etc., which cause diseases to humans and animals (Barker and Murrell, 2004), and they have the ability to transmit pathogens vertically through eggs (Blinick et al., 2017).

Ticks molt after blood-feeding in each life cycle, and it is a very complex process. Ticks have a variety of symbionts; the common symbionts found in different ticks are those belonging to the genus Rickettsia in the hard tick, and the genus Coxiella in the R. sanguineus, and the genus Francisella in the soft tick (Zhang et al., 2017; Ben-Yosef et al., 2020). Compared with insects such as mosquitoes and flies, there are few studies on the endosymbionts of R. sanguineus and some studies have investigated the biological specificity of the symbionts of Ixodes tick (Jasinskas et al., 2007; Zhong et al., 2021). Each study used different antibiotics and administration methods, and determined bacterial load and phenotypic characterization in different ways. This study aimed to determine changes of gut microbiota in R. sanguineus under different levels of starvation stress. Ticks can survive in the wild for months or even years without food, which leads to the increase in tick-borne diseases (Zhong et al., 2021). Starvation stress affects the physiological response of organisms, Coxiella is the most common maternally inherited endosymbiont, known for its ability to provide B vitamins and cofactors to ticks, and has an impact on tick development, fecundity and metabolism (Zhong et al., 2021). We hypothesized that there would be significant changes in the gut microbiota of the tick at different starvation periods, which ultimately reduced the number of provided nutrients and hindered development. We first subjected adult ticks to starvation stress for different periods to test this hypothesis. We then investigated the effect of symbiotic elimination on developmental timing and female reproductive success after nymph feeding. Bacterial diversity in whole-body samples of adult ticks at different starvation periods was also assessed.

2 Materials and methods

2.1 Tick collection and grouping

A total of 578 ticks were collected from the stray animal shelter in Haikou City, China. All ticks were identified morphologically to species level using existing taxonomic keys, followed by molecular analysis (Latrofa et al., 2014). Unfed female and male ticks were used in this study. They were first washed once in 70% ethanol and rinsed three times in sterile water. The ticks were kept under laboratory conditions in an incubator at 27.5°C and 90% relative humidity until needed for experiments. New Zealand white rabbits were used for blood-feeding larvae and nymphs. New R. sanguineus nymphs were assigned into six groups, named unfed, unfed1, unfed3, unfed4, unfed6, and fed. Unfed group nymphs were collected for testing immediately after molting. Groups unfed1, unfed3, unfed4, and unfed6 nymphs were collected for testing after 1, 3, 4, and 6 months starvation, respectively. Group fed nymphs were tested after blood feeding without starvation. Each group had 10–14 nymphs, and the experiment was repeated three times.

2.2 DNA extraction and PCR amplification

The nymphs collected from each group were washed with 75% alcohol and sterile water, and dissected to remove the mouthparts and four pairs of legs. The remaining bodies were pooled for DNA extraction for each group. The microbial DNA was extracted using the HiPure Soil DNA Kits (Magen, Guangzhou, China) according to the manufacturer’s protocols. The 16S rDNA target region of the ribosomal RNA gene was amplified by PCR (95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min) using primers listed in Supplementary Table S1 in the supplementary information (Guo et al., 2017). PCR reactions were performed in a triplicate 50 μL mixture containing 10 μL of 5 × Q5@ Reaction Buffer, 10 μL of 5 × Q5@ High GC Enhancer, 1.5 μL
of 2.5 mM dNTPs, 1.5 µL of each primer (10 µM), 0.2 µL of Q5® High-Fidelity DNA Polymerase, and 50 ng of template DNA.

2.4 Bioinformatics analysis

All purified amplicons were pooled in equimolar concentrations and paired-end sequenced on an Illumina PE250 platform. Bioinformatics analysis (OTU, community composition analysis, Indicator species analysis, α-diversity, β-diversity, and function prediction) of the raw data was performed using various software, FASTP (Chen et al., 2018) (version 0.18.0), FLSAH (Salzberg, 2011) (version 1.2.11), UPARSE (Edgar, 2013) (version 9.2.64), Krona (Ondov et al., 2011) (version 2.6), R package (Wickham and Boussiala, 2020) (version 2.2.1), QIIME (Caporaso et al., 2010) (version 1.9.1), Muscle (Edgar, 2010) (version 3.8.31), FastTree (Price et al., 2010) (version 2.1), Tax4Fun (Asshauer et al., 2015) (version 1.0). All sequencing and bioinformatics analyses were performed using the Omicsmart online platform (http://www.omicsmart.com).

2.5 Tissue tropism of Rh. sanguineus

Relative densities of Coxiella-like symbionts in the tissue sample of the gonads, Malpighian tubules, salivary glands, midgut, and trunk (remaining parts) were analyzed using the SYBR green or fluorescence quantitative PCR (qPCR) approach as previously described (Lalzar et al., 2012). Briefly, qPCR was used to detect the expression levels in different tissues using fluorescence dye SYBR Green I (Roche Molecular Biochemicals, Mannheim, Germany). The reaction system consisted of 5 µL of qPCR master mix, 0.5 µL of upstream and downstream primers, 1 µL of template cDNA, and 3 µL of ddH2O. Real-time amplification detection was performed using Roche’s Lightcycler 96. The standard procedure of two-step PCR amplification was adopted for the reaction: 95°C for the 20 s, 95°C for 5 s, and 58°C for 30 s, a total of 40 cycles. Three replicates were set for each sample, and the primers used in the experiment were shown in Supplementary Table S2.

2.6 Antibiotic injection for ticks

To obtain Rh. sanguineus with reduced Coxiella burden, we microinjected blood fed nymphs with oxolinic antibiotic at a dose of 30 mg/mg tick body weight and subsequently recorded the effect of nymphal and female development on health status. The selection of antibiotics was based on their previously established effectiveness against C. burnetti (Jabarit-Aldighieri et al., 1992; Gikas et al., 1998). Another aspect is their inhibitory effect on Coxiella in other ticks (Ninio et al., 2015). These studies highlighted oxolinic, rifampicin, and tetracycline as suitable candidates with substantial effects on Coxiella or Coxiella-like organisms. All drugs are broad-spectrum antibiotics that stop bacterial replication by interfering with DNA, RNA, and protein synthesis. The stock solution was diluted with sterile saline (0.9% NaCl, pH 6.5; oxolinic and tetracycline) or 1/5 v/v dimethyl sulfoxide rifampicin), adjusted to a final concentration of 7 mg/mL. Oxolinic was acidified and solubilized with hydrochloric acid (1:700 v/v normal saline: hydrochloric acid, pH 6.0), and all solutions were prepared and processed on a clean bench to ensure sterility. Our experiments show that different antibiotics have different potency against Coxiella and suggest that nymph developmental timing is affected differently by the antibiotics we tested. Subsequent experiments continued with the most potent antibiotic (oxolinic) and estimated the effect of antibiotics on inhibiting nymph body weight, blood feeding rate, and post-feeding development (time to adult molting). To determine the effect of Coxiella on nymph development, new nymphs were randomly assigned to different groups with similar mean weights and variances. Within the next 36–48 h, the ticks were microinjected (Hamilton microinjector 2.5 µL) in sterile physiological saline solution with or without antibiotics and placed in the artificial culture at 27.5°C and 90% RH. In the box, the molting of nymphs was recorded every 24 h until all molted ticks became adults. Subsequent experiments were continued using an antibiotic (oxolinic) against Coxiella. The effects of inhibition of Coxiella on nymph body weight, and post-feeding nymph development (time to adult molting) were evaluated. To determine the effect of Coxiella spp. on nymphal development, fully saturated nymphs were isolated from New Zealand white rabbits (collected at two subsequent 24-h intervals), weighed to the nearest 0.05 mg, and then randomly assigned to average body weight and in groups with similar variances. Over the next 24 h, nymphs were microinjected with 1.5 µL of sterile saline solution with or without antibiotics, then placed in 50 ml sterile centrifuge tubes perforated with caps and kept in the dark climate chamber (27 ± 1°C and (85 ± 5%) relative humidity to complete the molting and development after blood feeding. During the following period, we recorded the molting of the nymphs every 24 h until all nymphs completed molting and became adults. These experiments were repeated twice.

For male-female mating and blood-feeding, molted female ticks were parasitized on New Zealand white rabbits. They were subsequently used to determine the effect of Coxiella on nymph blood-feeding rate, saturated blood weight, adult fecundity (hatching rate), and post-hatch larval blood-feeding rate. Rh. sanguineus was released to the host skin after the antibiotic treatment, the feeding period of female ticks lasted for 8–18 days.

2.7 Data analysis

The diversity of starvation stress time microbial communities with a supplied diet was evaluated by 16S
rRNA gene sequencing. After sequencing, we first filtered the low-quality reads from raw reads obtained, assembled the paired-end reads into tags, and then filtered the tags. The obtained data is called Clean tag. Next, clustering is performed based on the Clean tags, the chimera tags detected during the clustering alignment were removed, and the obtained data is the Effective tag. Data preprocessing statistics and quality control were shown in Supplementary Table S3. After obtaining OTUs, the overall characteristics of each sample OTU, low abundance OTU, Tags annotation, and other information were statistically summarized based on OTU’s abundance information and species annotation information. The results were shown in Table 1.

All the data were tested for normality using the Shapiro-Wallis test. The differences between three or more groups were determined using the Kruskal-Wallis test. Other comparisons were performed using Mann Whitney test or two-tailed t-test depending on Gaussian distribution. The values were statistically significant when \( p < 0.05 \). All statistical calculations were performed using GraphPad Prism software (version 7) except the effect size, calculated using the Cohen’s \( d \).

### 2.8 Ethics statement

The use of experimental animals was reviewed and approved by Hainan University Institutional Animal Care and Use Committee (HNUAUCC-2021-00095).

### 3 Results

#### 3.1 Effects of starvation stress on microbial communities in ticks

After removing the low quality or no biological significance tags, a total of 1,963,500 effective tags were acquired from the 18 samples analyzed. An average of 109,083 effective tags was covered for each sample. The effective ratio of all samples averaged 84.81% (ranging from 82.15% to 86.1%) (Supplementary Table S1). The diversity index curve that with the extension of starvation time and the curves of all samples were gradually flattened and saturated, indicating that the sequencing quality of all samples was reliable. The \( \alpha \)-diversity indices including ACE, Shannon, Simpson, and Sob are depicted in Figure 1. The above four alpha diversity indices of the unfed group were higher than those of the fed group. Notably, species richness in the unfed group-1 was higher after the starvation treatment.

#### 3.2 Microbial composition in ticks

Different bacteria of 30 phyla, 86 classes, 129 orders, 234 families, 485 genera, and 149 species were identified in all ticks. Figure 2, shows that the total number of Proteobacteria, Actinobacteria, and Firmicutes was more than 80%. Therefore, the focus is on these three particular phyla. At the genus level, the majority of species belonged to Coxiella, followed by Staphylococcus, Brevibacterium, Acinetobacter, Corynebacterium, Pseudonocardia, Lactobacillus, Saccharopolyspora, and other unclassified bacteria.

### Table 1 Statistics of tags and OTUs.

| Sample ID | Total tags | Taxon tags | Unclassified tags | Singleton tags | OTUs |
|-----------|------------|------------|------------------|----------------|------|
| Fed-1     | 110319     | 103558     | 0                | 6,761          | 227  |
| Fed-2     | 111034     | 105161     | 0                | 5,873          | 170  |
| Fed-3     | 104216     | 99075      | 0                | 5,141          | 167  |
| Unfed-1   | 111450     | 105474     | 0                | 5,976          | 178  |
| Unfed-2   | 113370     | 107776     | 0                | 5,594          | 153  |
| Unfed-3   | 102057     | 97371      | 0                | 4,686          | 141  |
| Unfed1-1  | 116223     | 107236     | 0                | 8,987          | 275  |
| Unfed1-2  | 113451     | 107712     | 0                | 5,739          | 313  |
| Unfed1-3  | 114760     | 107192     | 0                | 7,568          | 231  |
| Unfed3-1  | 108206     | 101606     | 0                | 6,600          | 151  |
| Unfed3-2  | 113693     | 106976     | 0                | 6,717          | 220  |
| Unfed3-3  | 110401     | 89921      | 0                | 20480          | 623  |
| Unfed4-1  | 108871     | 102902     | 0                | 5,969          | 221  |
| Unfed4-2  | 102971     | 97678      | 0                | 5,293          | 200  |
| Unfed4-3  | 102743     | 97416      | 0                | 5,327          | 152  |
| Unfed6-1  | 104089     | 97620      | 0                | 6,469          | 231  |
| Unfed6-2  | 104333     | 98382      | 0                | 5,951          | 215  |
| Unfed6-3  | 111313     | 104597     | 0                | 6,716          | 262  |
| Avg       | 109083     | 102091     | 0                | 6,991          | 229  |
3.3 Prediction of functional profiles of tick microbiota

The Tax4Fun algorithm in the methods section revealed the functional profile of the gut microbiota of *R. sanguineus* in each group. Based on the Silva database, to better describe the changes in functional gene-related pathways in different groups, the heat map of the predicted function map of 16S rRNA genes obtained by Tax4Fun showed the OTU annotation information of each functional level of KEGG (Figure 3). All groups showed significant changes according to KEGG functional analysis. As shown in Figure 3A, an overview of the functional distribution can be organized into six typical signaling pathways, including metabolism, environmental information processing, genetic information processing, cellular processes, human diseases, and tissue systems. As shown in Figure 3A, metabolism,
environmental information processing, and genetic information processing were strongly affected in this study. The remaining three key signaling pathways were also affected to some extent.

Further analysis showed that starvation stress led to changes in all metabolic pathways. As shown in Figure 3B, starvation significantly enhanced carbohydrate metabolism, membrane transport, amino acid metabolism, cell growth, and death and cell motility compared to the fed group, suggesting that it impaired the function of translation, metabolism of cofactors and vitamins, and energy metabolism. In addition, compared with the fed group, the purine metabolism, ammonia synthesis, dimethyl ether metabolism, starch, and sucrose metabolism, amino sugar metabolism, ribosome synthesis, peptidoglycan biosynthesis, and synthesis of the Unfed3, Unfed4, and Unfed6 groups signaling pathways were upregulated. Notably, in the Unfed6 group, all other signaling pathways except arginine and proline metabolism, porphyrin and chlorophyll metabolism, oxidative phosphorylation, pyruvate metabolism, and nitrogen metabolism were upregulated. ABC transport, purine metabolism, pyrimidine metabolism, peptidoglycan biosynthesis, arginine, proline metabolism, RNA degradation, bacterial secretion system, glycine, serine, and threonine metabolic pathways were significantly enhanced in the Unfed3 group. The acid metabolism pathway was significantly enhanced. The above results further showed that after starvation stress, the changes of microbes and signaling pathways in different parts of the gut of Rh. sanguineus were altered.

3.4 Analysis of 16S rRNA gene tissue tropism

Coxiella spp. density in different organs dissected from Rh. sanguineus ticks collected from the field were determined by qPCR. Estimated bacterial densities differed in different organs of females and males are shown in Figures 4A,B. The highest density of Coxiella spp. is found in female gonads. This number of bacteria was 1.4 times higher than in female Malpighian tubules, although there was no significant difference ($p > 0.05$) (Figure 4A). The densities in these two organs accounted for 97% of the total number of Coxiella spp. in female anatomical organs (Figure 4B). The main difference between male and female ticks is the almost complete absence of Coxiella in the male gonads (Figure 4A). Although higher variation was
found, on average, 97% Coxiella in the males were present in the Malpighian tubules (Figure 4C). Interestingly, the densities of Coxiella in the Malpighian tubules of male and female ticks were similar ($p > 0.05$).

### 3.5 Effects of ofloxacin injection on ticks

Our experimental results showed that the molting time of female ticks was slightly prolonged after ofloxacin treatment, while the molting time of male ticks had little effect (Figures 5A,B).

On average, these female ticks finished feeding and left the host at 13.57 ± 0.43 days in New Zealand white rabbits, which was about 3 days later than the female ticks not injected with ofloxacin (the time of full blood was 16.57 ± 0.61 days), which was significantly higher in the untreated group ("Oflo": $t = 4.017$, $DF = 12$, $p < 0.0017$, Figure 6A). Some ticks injected with ofloxacin could not get fully engorged, even if they stayed on the rabbit skin for a week ($n = 7$). The weight of female ticks injected with ofloxacin was approximately 76.6% of the final saturated bodyweight of the untreated group (214.2 ± 2.239 mg and 164.7 ± 4.779 mg, respectively); the weight of female ticks removed from Coxiella at the end of feeding was significantly lower than that of the untreated group containing saline-injected female ticks ("Oflo": $t = 4.017$, $DF = 12$, $p < 0.0017$, Figure 6B). Some female ticks were still alive after full blood feeding and eventually died naturally without laying eggs ($n =$...
3) or shortly after leaving the host (n = 1). This phenomenon rarely occurs in symbiotic female ticks, suggesting that inhibition of *Coxiella* causes a significant stress on female tick feeding. All other health-related data that we quantified during the experiment were negatively affected. Compared with the normal saline group, the hatching rate of eggs in ofloxacin injected group was significantly lower ("Oflx" t = 8.968, DF = 20, p < 0.0001, Figure 6C). After the nymphs successfully molted into adults, they continued to lay eggs after full blood feeding, and calculated the changes in the larval blood-sucking rate. It was found that the blood-sucking rate of the ofloxacin injection group was 5.94% lower than that of the control group ("Oflx" t = 3.588, DF = 20, p < 0.0018, Figure 6D).

### 4 Discussion

The purpose of this study was to study the microbiome regulation of *Rh. sanguineus* under starvation stress. With the prolongation of starvation stress, the abundance of *Coxiella* gradually decreased. After using ofloxacin to eliminate *Coxiella* in the nymph, the blood-sucking rate, fecundity, and development were all affected to varying degrees. qPCR results showed that the density of *Coxiella* in the Malpighian tubules was significantly higher than those in all other organs. The main difference between male and female ticks was the almost absence of *Coxiella* in the male gonads. Although higher variability was found, on average, 97% of male *Coxiella* were present in the Malpighian tube. The density of *Coxiella* in the Malpighian tubules was similar in male and female ticks after ofloxacin injection. Pre-molt development time of female and male ticks, feeding period, engorgement weight, fecundity of the adults, and the feeding rate of the newly hatched larvae were all affected to varying degrees. Although starvation stress significantly reduced the abundance of the *Coxiella* flora, it increased relative abundance of microbial diversity in *Rh. sanguineus*.

The results of the subsequent antibiotic experiments further confirmed the effect of *Coxiella* on the development and reproduction of *Rh. sanguineus*. We did not find a dose-dependent effect of ofloxacin on nymph developmental timing, suggesting that ofloxacin has a negligible direct effect on ticks. In addition, the uniqueness of our study is...
the starvation stress of *Rh. sanguineus* initially demonstrated. We find that its starvation tolerance was as long as 6 months, which was not mentioned in other studies. Our findings of the microbiota pathway distribution and functional prediction of *Rh. sanguineus* under starvation stress suggest that the development and reproduction of *Rh. sanguineus* which are affected by the symbiotic *Coxiella.* Studying the distribution of symbionts and the mechanisms of nutrient metabolism may be a powerful option for controlling ticks and the pathogens that they can carry. The starved ticks had fewer endosymbionts in the midgut and salivary glands, and more in the Malpighian tubule and gonads, suggesting that either the tick is digesting the symbionts for nutrients, or the microbes are dying/moving on their own. A high density of *Coxiella*-like endosymbionts in Malpighian tubules supports a nutritional role for these symbionts, as Malpighian tubules are involved in excretion and osmoregulation. We postulate that Malpighian tubules are key organs for the nutritional symbiosis, notably the synthesis of B vitamins by *Coxiella.*

In summary, this study showed the microbial community of *Rh. sanguineus* survived for 6 months under starvation conditions in the laboratory. Therefore, the microbial community in its body changed significantly, and the symbiotic microorganism *Coxiella* regulated the development of *Rh. sanguineus* and reproduction. These findings may provide insight into implications for understanding of nutritional contribution in ticks and its consequences for tick biology and tick-borne diseases.

**Data availability statement**

The data presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

**Ethics statement**

The animal study was reviewed and approved by the Hainan University Institutional Animal Care and Use Committee (HNUAUCC-2021-00095).

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Conceptualization, QH and JZ; writing—original draft preparation, LA and BB; performing the experiments and analyzing the results, LA, DL, and PS; supervision, JZ and QH; project administration, CL; funding acquisition, QH; Revising and editing, BB and QH. All authors have read and agreed to the published version of the manuscript.

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**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/fphys.2022.932130/full#supplementary-material

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