

ORIGINAL ARTICLE

The bacterial microbiome of *Dermacentor andersoni* ticks influences pathogen susceptibility

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Ticks are of medical importance owing to their ability to transmit pathogens to humans and animals. The Rocky Mountain wood tick, *Dermacentor andersoni*, is a vector of a number of pathogens, including *Anaplasma marginale*, which is the most widespread tick-borne pathogen of livestock. Although ticks host pathogenic bacteria, they also harbor bacterial endosymbionts that have a role in tick physiology, survival, as well as pathogen acquisition and transmission. The goal of this study was to characterize the bacterial microbiome and examine the impact of microbiome disruption on pathogen susceptibility. The bacterial microbiome of two populations of *D. andersoni* with historically different susceptibilities to *A. marginale* was characterized. In this study, the microbiome was disrupted and then ticks were exposed to *A. marginale* or *Francisella novicida* to determine whether the microbiome correlated with pathogen susceptibility. Our study showed that an increase in proportion and quantity of *Rickettsia bellii* in the microbiome was negatively correlated to *A. marginale* levels in ticks. Furthermore, a decrease in *Francisella* endosymbionts was associated with lower *F. novicida* infection levels, demonstrating a positive pathogen–endosymbiont relationship. We demonstrate that endosymbionts and pathogens have varying interactions, and suggest that microbiome manipulation may provide a possible method for biocontrol by decreasing pathogen susceptibility of ticks.

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Introduction

Ticks are obligate hematophagous arthropods that feed on vertebrates and are of medical importance owing to their ability to inflict harm to humans and animals (Jongejan and Uilenberg, 2004). In particular, ticks transmit pathogens throughout all continents and are labeled as principle vectors of zoonotic pathogens (Clay and Fuqua, 2010; Ahantarig *et al.*, 2013). In fact, >15 new tick-borne bacterial pathogens have been discovered since Lyme disease was characterized in 1982 (Jongejan and Uilenberg, 2004).

Dermacentor andersoni, the Rocky Mountain wood tick, has been labeled a ‘veritable Pandora’s box of disease-producing agents’ (Cooley, 1938) and transmits several pathogens, including *Rickettsia rickettsii* and *Francisella tularensis*. It is also a vector of *Anaplasma marginale*, the agent of anaplasmosis,

and the most widespread tick-borne pathogen of livestock worldwide (Kocan *et al.*, 2003). Economic losses due to anaplasmosis in cattle are estimated to be >\$300 million per year in the United States (Rochon *et al.*, 2012).

Many microorganisms colonize ticks, with the majority being non-pathogenic endosymbionts. The endosymbiont community has a role in tick physiology, survival, and importantly, pathogen acquisition and transmission (Clay and Fuqua, 2010). *Amblyomma americanum* exhibits decreased fitness in all life stages when the microbiome is manipulated by antibiotic exposure (Zhong *et al.*, 2007). In insects, bacterial species that are required for survival and are maintained within the population transovarially are classically considered to be primary endosymbionts, whereas secondary endosymbionts are beneficial or non-threatening and colonize insects through environmental exposure (Ahantarig *et al.*, 2013). Currently, the extent to which environmental conditions shape the microbiome is not known. There have been varied reports in the literature of environmental impact on the tick microbiome, which suggests the idea that there is not a conserved microbiome. For example, geographic location has been shown to affect the microbial composition of *Ixodes ricinus* ticks

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(Halos *et al.*, 2010), however, there was not a habitat-related difference in the microbiome of *Dermacentor variabilis* and *Ixodes scapularis* ticks (Hawlena *et al.*, 2012).

The bacterial microbiome, in addition to other functions, is thought to aid tick defense against pathogens. Evidence of endosymbiont-pathogen-inhibiting abilities include the inhibition of *R. rickettsii* transovarial transmission by *Rickettsia peacockii* (Telford, 2009). Interestingly, geographically distinct populations of *D. andersoni* have been shown to experience intraspecific variation in pathogen acquisition: *D. andersoni* populations from Lake Como, MT, USA have lower *Anaplasma marginale* acquisition rates (12–30%) than populations from Burns, Oregon (54–62%) (Scoles *et al.*, 2005). In addition, *A. marginale* acquisition rates vary over time, within a location. We hypothesize that the tick microbiome affects pathogen susceptibility, resulting in differences in pathogen acquisition between tick populations. We tested this hypothesis by examining the microbiome of two geographically distinct populations of *D. andersoni*, altering the microbiome, and testing for pathogen acquisition with two pathogens, *A. marginale* and *Francisella novicida*.

Materials and methods

Tick collection and colonization

Questing adult *D. andersoni* were collected by flag and drag techniques at sites in Burns, Oregon and Lake Como, Montana (Scoles *et al.*, 2005). Field-collected ticks were used to establish laboratory colonies, which were reared and maintained at the Holm Research Center at the University of Idaho in Moscow, ID, USA (Washington State University IACUC #04440-004 and University of Idaho IACUC #2013-66) (Scoles *et al.*, 2005). Unless there is an ongoing pathogenic infection, the host blood meal does not affect the arthropod microbiome (Hawlena *et al.*, 2012). Furthermore, we screened any bovine blood used in our experiments for the presence of bacteria before colony feeds by running PCR with universal 16S primers.

For tick microbiome analysis (Figure 1), a cohort of adult F1 or F2 male ticks from each colony was fed on a Holstein calf and dissected to collect midguts (MG) and salivary glands (SG) for genomic DNA isolation. Adult males were analyzed given that they are the primary source of *A. marginale* transmission, as the males move from infected to naive hosts in search of mates (Scoles *et al.*, 2005). In addition, MG and SG are the tissues colonized by *A. marginale* during acquisition and transmission, respectively (Ueti *et al.*, 2007).

DNA preparation and sequencing

F1 and F2 generation adult male ticks were fed for 7 days and then dissected within 24 h. Before

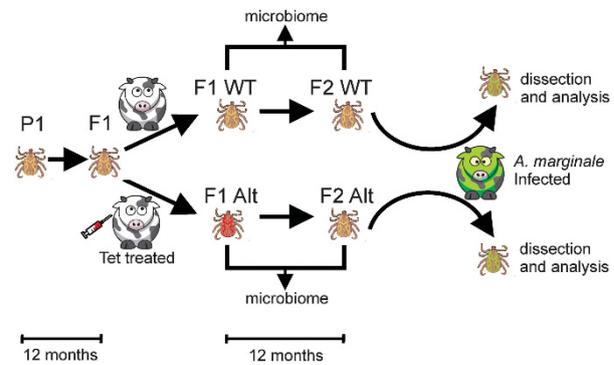


Figure 1 The experimental design for microbiome analysis and pathogen acquisition. P1 ticks were collected from the field and reared one generation to produce F1 adults. F1 adult male ticks were exposed to a broad-spectrum antibiotic (oxytetracycline; Tet treated) by feeding on an antibiotic-treated calf to disrupt the bacterial community and to produce altered F2 adults (F2 Alt; red-colored ticks). A cohort of F1 adults were reared identically to F2 Alt ticks, but were not exposed to the antibiotic producing the F2 WT adults. F2 (WT and Alt) adult male ticks (green-colored ticks) were fed on an *A. marginale*-infected calf (green-colored calf) and cohorts were collected at 2 and 6 days, dissected to collect MG and SG, which were used to assess pathogen infection. Larvae were fed on laboratory rats and nymphs and adults were fed on cattle. The 12-month scale represents the time it takes to rear one generation of ticks under laboratory conditions.

dissection, the ticks were surface sterilized and all dissection tools were sterilized between each dissection (Scoles *et al.*, 2005). Tick MG and SG were dissected and pooled in groups of 30 with three biological replicates. Tissues were stored in Cell Lysis Solution (Qiagen, Valencia, CA, USA) and Proteinase K (1.25 mg ml^{-1}). Genomic DNA was isolated using the PureGene Extraction kit (Qiagen) according to the manufacturer's specifications (Scoles *et al.*, 2005). In total, 16S recombinant DNA was amplified using Platinum Pfx DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and the primers described below at an annealing temperature of 54°C . Each sample was amplified in at least three technical replicates with the same sample barcode. To pool our samples, we used barcoded sample-specific primers following the Amplicon Fusion Primer format (Andreotti *et al.*, 2011). To target as many 16S variable regions as technically possible, we used modified universal primers 27F (AGAGTTTGATCMTGGCTCAGAACG) and 1435R (CGATTA CTAGCGATTCCRRCTTCA) (Turner *et al.*, 1999; Lane, 1991). The DNA concentration of each sample was measured with a bioanalyzer (Agilent, Santa Clara, CA, USA) and samples were pooled in equimolar amounts. Samples were submitted to Washington State University's Sequencing Core for Pacific Bioscience (PacBio, Menlo Park, CA, USA) Circular Consensus Sequencing.

Sequence analysis

Sequence reads were analyzed using the Ribosomal Database Program (Cole *et al.*, 2009) and CLC Genomics Workbench. Raw sequence data were

filtered by PacBio software according to the expected sequence size range and 99% accuracy. After filtering, reads were blasted against National Center for Biotechnology Information BLASTn database. Blast results were filtered at a minimum of 1275 bp in length and 98% identity. Reads that were below 98% identity were reported at genus level, whereas 98% and above were classified at the species level (Jones *et al.*, 2009; Bonnet *et al.*, 2014; Budachetri *et al.*, 2014). Operational taxonomic units that accounted for 1% or less of the total proportion of the microbiome were grouped together in a 'rare' category. The average number of reads/sample after filtering was 3444 sequences, which satisfied a rarefaction curve allowing us to be confident that all operational taxonomic units were captured. Raw microbiome sequence data were deposited in the sequence read archive at National Center for Biotechnology Information (SRR2049110—2049116 and SRR2049119—2049159).

Pathogens used in this study

The Saint Maries strain of *A. marginale* is the prototypical efficiently tick transmitted strain and the type strain for *A. marginale* (Dumler *et al.*, 2001). The genome of the St Maries strain has been completely sequenced, and this strain has been used in numerous studies of vector efficiency (Futse *et al.*, 2003; Brayton *et al.*, 2005; Scoles *et al.*, 2005). *F. tularensis* subspecies *novicida* strain U112 is a rare human pathogen, causing mild disease. Our interest in this pathogen is that it behaves similarly to *F. tularensis* and provides a model system to study tick transmission (Reif *et al.*, 2011).

Microbiome disruption

The microbiome was disrupted to study the consequences of an altered microbiome on pathogen acquisition. To alter the microbiome, a cohort of F1 ticks from both Burns and Lake Como colonies were fed on either antibiotic-treated calves (which produced 'Alt' microbiomes) or untreated calves (which produced wild-type 'WT' microbiomes). The antibiotic-treated calves received a therapeutic dose (11 mg kg⁻¹ of body weight) of Liqueamycin LA-200 oxytetracycline injections on -4, -1, +3 and +5 days post application of ticks, whereas untreated calves did not receive any injections. This broad-spectrum antibiotic is commonly used to treat a broad range of bacterial infections in cattle, as we intended to broadly target the microbiome. Ticks were grouped into an 'experimental cohort', 100 male ticks that were dissected and processed for sequencing following 7 days of feeding, or a 'colony cohort' that included 100 males and 100 females that were allowed to feed to repletion. In both colony cohorts, females were allowed to oviposit to continue a second generation of the WT and Alt

microbiomes (F2 generation, Figure 1). Note that a single discreet antibiotic treatment was used, and the F2 Alt generation arose from F1 adults that were exposed to antibiotics. The F2 ticks were not subjected to antibiotics.

Acquisition of A. marginale

F2 WT and Alt adult male ticks from Burns and Lake Como cohorts were fed on a Holstein calf that was persistently infected with *A. marginale* St. Maries strain (6.55 × 10⁵ organisms ml⁻¹ of whole blood). Ninety ticks from each of the four treatment groups were fed in a group-specific stockinette patch. After 12 h all unattached ticks were removed. Cohorts of each group were removed from the calf at 2, 4 and 6 days post application. After removal, ticks were held for 7 days at 26 °C and 98% relative humidity to allow for digestion of the blood meal and then the MG and SG were dissected. Day 2 represents the minimal amount of time that it takes for the tick to become infected with *A. marginale*, whereas day 6 represents ticks that are ready for transmission feeding (Eriks *et al.*, 1993).

Genomic DNA from acquisition fed ticks was extracted using the PureGene Extraction kit (Qiagen). Pathogen infection rates and levels were analyzed by quantitation of the *A. marginale* single-copy gene, *msp5*, using a Bio-Rad Digital Drop PCR (ddPCR; Hercules, CA, USA) machine. The primer set included *msp5_for2* (CCGAAGTTGTAAGTGAGGG), *msp5_rev2* (CTTATCGGCATGGTCGCC) and *msp5_probe* (CCT CCGCGTCTTTTCGACAATTTGGTT). Thermocycling conditions were as follows: 10 min denaturation at 95 °C; 39 cycles of 94 °C for 30 s, 60 °C for 60 s; and a final cycle of 98 °C for 10 min. Sample preparation and ddPCR protocols followed the manufacturer's recommendations. Although there is a physical size difference between Burns and Lake Como ticks, *A. marginale* infection rates and levels are independent of size (Scoles *et al.*, 2005).

Acquisition of F. novicida

To determine whether the tick microbiome affected the ability of ticks to acquire *Francisella* pathogens, WT and Alt nymphs from F2 Burns and Lake Como were fed on *F. novicida* U112-infected mice as previously described (Reif *et al.*, 2011). To minimize host-to-host variation, WT and Alt ticks from the same geographic population fed on the same mice, but in separate capsules. Replete nymphs were collected after feeding, incubated at 25 °C and allowed to molt to adults. Midguts were individually harvested from adult ticks, homogenized and the resulting lysate plated (Reif *et al.*, 2014). Enumeration of colony forming units (CFU) was used to calculate the *F. novicida* infection level in tick MG. Mouse bacteremia was determined by plating blood (Reif *et al.*, 2011).

Quantitative analysis of Rickettsia bellii

To quantify *R. bellii*, *rickA* copy numbers were measured using SYBR Green quantitative PCR (Invitrogen) of Lake Como WT and Alt in F1 and F2 ticks. The quantity of *R. bellii* was determined using qRbRickAF2 (5'-TACGCCACTCCCTGTGTCA-3') and qRbRickAR (5'-GATGTAACGGTATTACACCAACAG-3') primers (Oliver et al., 2014). The bacterial quantity was measured in F1 and F2 MG and SG of the pooled samples that were used for PacBio sequencing. The quantities of *R. bellii* were used to verify proportional changes of *R. bellii* within the microbiome when comparing WT and Alt compositions, as well as the differences in the microbiome between F1 and F2 generations.

Statistical analysis

Methods for analyzing microbiome data vary greatly and many publications have used paired *t*-tests for their simplicity and applicability to a wide range of data (Zhong et al., 2007; Clay et al., 2008; Carpi et al., 2011; Budachetri et al., 2014; Bonnet et al., 2014). We used a generalized linear mixed (GLM) model, as well as traditional pairwise comparisons for the *A. marginale*, *R. bellii* and *F. novicida* data. By using both types of statistical analyses we can compare our data with previous studies as well as the two types of statistical methods.

The following data were analyzed with GLM models in the Glimmix Procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA): microbiome, *A. marginale* acquisition, *F. novicida* and *R. bellii*. Within the various models, fixed effects included population (L), treatment of calves (T), day of tick feeding (D), organ (O) and generation of tick (G) with a random effect of replicate. Replicate did not account for any variation and was removed from all models. In addition, all models were reduced in a stepwise manner starting with highest order interactions that were not significant. The Tukey–Kramer procedure for unbalanced data were used for all pairwise comparisons.

Microbiome data were transformed to proportion of endosymbiont per pooled sample of ticks to reduce the heterogeneity of variances. In addition, a single count was added to all *R. bellii* counts prior to being transformed to allow for comparison of the populations, as Burns had only one tick pool with a non-zero count. Endosymbiont data were described using:

$$Y_{ijkl} = \mu + L_i + T_j + O_k + L_iO_k + e_{ijkl},$$

A. marginale acquisition data were described using:

$$Y_{ijklm} = \mu + L_i + T_j + L_iT_j + D_k + L_iD_k + T_jD_k + O_l + L_iO_l + T_jO_l + D_kO_l + L_iT_jD_k + e_{ijklm},$$

F. novicida data were described using:

$$Y_{ijk} = \mu + L_i + T_j + e_{ijk}$$

R. bellii data were described using:

$$Y_{ijkl} = \mu + G_i + T_j + G_iT_j + O_k + T_jO_k + e_{ijkl},$$

Spearman rank-order correlations (SAS9.4) were calculated between *A. marginale* and *R. bellii* within F2 Lake Como ticks. One-way analysis of variance within SPSS (IBM Version 20) was used to calculate pairwise comparisons to analyze statistical differences between and within groups' pathogen infection rates and levels of *A. marginale* acquisition, as well as comparisons of tick-fitness measurements. Generalized linear mixed-model output has been included in Supplementary File 1 (Supplementary Tables 1–7).

Results

Bacterial microbiome characterization

We compared tick microbiomes across populations and tissue types. The bacterial composition of both tick populations varied by tissue type. In the population from Burns, the MG had two endosymbiont groups, a mixture of *Francisella spp.* (20%) and *Francisella*-like endosymbiont (FLE; 61%), that made up 81% of the microbiome, whereas the SG microbiome was composed mainly of *Arsenophonus spp.* (95%; Figure 2a). The MG microbiome of the Lake Como population was composed primarily

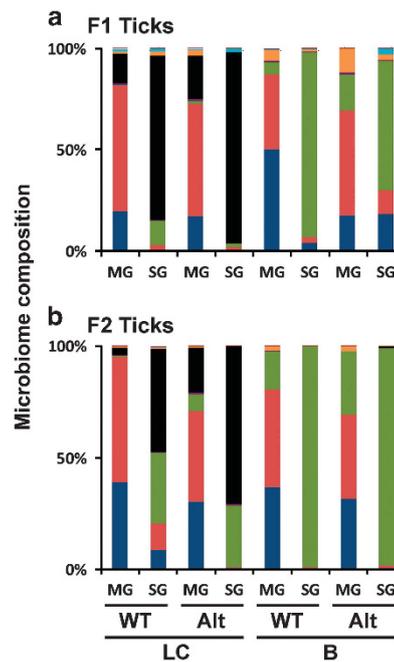


Figure 2 Microbiome of *D. andersoni* ticks in wild-type and altered populations from Montana and Oregon. The bacterial composition of the midgut (MG) and salivary glands (SG) of adult male Burns (B) and Lake Como (LC) ticks was characterized in (a) F1 ticks and (b) F2 progeny ticks. Species/genera detected included *Francisella spp.* (blue), *FLE* (red), *Arsenophonus spp.* (green), *Rickettsia spp.* (purple), *R. bellii* (black), *R. peacockii* (orange), rare (teal) and unclassified (dark red).

of FLE (60%), *Francisella* spp. (20%), and a small proportion of a *R. bellii* (16%), whereas the SG microbiome was composed primarily of *R. bellii* (82%) and *Arsenophonus* spp. (11%).

The GLM model found that all endosymbionts varied in the overall proportion of total endosymbionts across both tick populations, except for *Francisella* spp. ($P > 0.21$) and rare ($P > 0.56$) endosymbionts (Figure 2a; Supplementary File 1 Supplementary Table 4). Burns ticks had a greater proportion of *Arsenophonus* spp. (45.7%; $P < 0.01$) and *R. peacockii* (5.1%; $P < 0.05$) compared with Lake Como ticks. A greater proportion of FLE (6.7%; $P < 0.05$), *R. bellii* (54.2%; $P < 0.01$) and *Rickettsia* (1.4%; $P < 0.02$) were found in Lake Como ticks compared with Burns ticks. In addition, the proportions of bacteria varied based on tissue type, except for *Rickettsia* ($P > 0.06$) and rare ($P > 0.06$) endosymbionts (Figure 2a). The proportion of FLE (54.9%; $P < 0.01$), *Francisella* spp. (31.6%; $P < 0.01$), *R. bellii* (33.8%; $P < 0.01$) and *R. peacockii* (4.9%; $P < 0.05$) was greater in MG compared with SG. SG had a greater proportion of *Arsenophonus* spp. (48.4%; $P < 0.01$) compared with MG.

Microbiome alteration

The complexities of the microbiomes were unchanged by antibiotic exposure; specifically no bacterial species were introduced or lost, although endosymbiont proportions shifted owing to antibiotic exposure (Figure 2a). Antibiotics altered the microbiome in the Burns population, with an increase in the proportion of *Arsenophonus* spp. (19.3%) and a decrease in the proportion of *Francisella* spp. (5.0%) and FLE (9.4%) in the SG. In contrast, in the Alt MG, the proportion of *Francisella* spp. and FLE decreased by 10.5% and 5.2%, respectively, and *Arsenophonus* spp. increased 2.5%. The response to antibiotic exposure in the Lake Como tick population was different than the response of the Burns tick population. The Lake Como population experienced an increase in the proportion of *R. bellii* by 12.3%, and a decrease in *Arsenophonus* spp. by 8.4%, in the SG owing to antibiotic treatment. The MG showed a decrease in the proportion of FLE (8.4%), whereas the proportion of *R. bellii* increased by 8.9% (Figure 2a), with antibiotics.

Ticks were reared one generation post disruption (F2 ticks) to evaluate whether the alteration to the microbiome was maintained. Burns Alt F2 ticks (Figure 2b) had an increased proportion in *Arsenophonus* spp. and decreased proportions in both *Francisella* spp. as well as FLE in the MG without any change in the SG as compared with WT Burns ticks. When comparing F2 Alt and WT Lake Como ticks the bacterial composition of the MG showed an increase in the proportion of *R. bellii* (15.2%) and *Arsenophonus* spp. (7.5%) and decreases in *Francisella* spp. (8.4%) and FLE (15.0%), whereas in the SG

there were decreases in the proportion of *Francisella* spp. (8.5%) and FLE (12.0%) and increased proportion of *R. bellii* (24.7%).

When the GLM model was applied to the data, FLE ($P < 0.02$) and *Francisella* spp. ($P < 0.02$) were in a greater proportions in F2 WT ticks compared with Alt ticks, regardless of the tick population or organ type, confirming observations in the unadjusted data. In addition, there were significant ($P < 0.02$) tick population by organ-type interactions for *Arsenophonus* spp., *R. bellii*, *R. peacockii* and rare endosymbionts. With *Arsenophonus* spp., Burns ticks had a much greater ($P < 0.01$) proportion in the SG (84.9% and 97.5%, F1 and F2, respectively) compared with Lake Como ticks (6.3% and 26.7%, F1 and F2, respectively). In addition, SG of Burns ticks had a greater ($P < 0.01$) proportion of *Arsenophonus* spp. than MG. In F1 ticks, *R. bellii* was proportionally different ($P < 0.01$) between Lake Como MG (20.6%) and SG (88.1%), MG between Burns (0.1%) and Lake Como (20.6%), and SG between Burns (0.2%) and Lake Como (88.1%). In F2 ticks, *R. bellii* was proportionally different ($P < 0.01$) between Lake Como MG (11.1%) and SG (60.9%) and SG between Burns (1.1%) and Lake Como (60.9%). In F2 ticks, *R. peacockii* had different ($P < 0.02$) proportions between MG of Burns (1.9%) and Lake Como (0.7%) and Burns MG (1.9%) and SG (0.1%). In F2 rare endosymbionts, MG had a different ($P < 0.03$) proportion in Burns (0.4%) compared with Lake Como (0.06%) and Burns MG (0.4%) compared with SG (0.02%).

A. marginale acquisition

For the WT and Alt adult male F2 ticks from Burns and Lake Como that were fed on a calf persistently infected with *A. marginale* the Alt ticks had significantly less *A. marginale* (4.42×10^3 *A. marginale*/tick) than WT ticks (7.22×10^3 *A. marginale*/tick; $P < 0.01$; Figure 4). Also, *A. marginale* increased over time, with the highest levels of *A. marginale* detected at day 6 ($P < 0.01$; Figure 4). All groups had high rates of *A. marginale* infection by day 2, without a significant difference between days or treatment, with the exception that Lake Como Alt ticks all had a significantly lower infection rate than Burns Alt ticks at day 6 (Supplementary File 1 Supplementary Table 8).

Tick populations had significantly different levels of acquisition of *A. marginale* ($P < 0.006$), with Burns ticks having greater *A. marginale* loads (7.32×10^3 *A. marginale*/tick) than Lake Como ticks (4.35×10^3 *A. marginale*/tick), irrespective of tissue or treatment (Supplementary File 1 Supplementary Tables 1). The Burns population did not have any significant differences between treatment groups on day 2 or 6 (Figure 3). Analysis of pathogen load in Lake Como ticks was significantly different between the WT and Alt groups on days 2 and 6 (Figure 3). In the Lake Como ticks, fewer *A. marginale* occurred in the

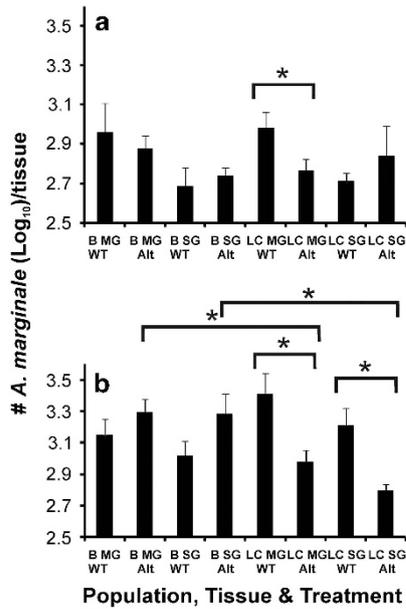


Figure 3 *A. marginale* infection load. *A. marginale* load in Lake Como (LC) and Burns (b) ticks is shown for day 2 (a) and day 6 (b) of acquisition feeding. The pathogen was quantified in the midgut (MG) and salivary glands (SG) using the single-copy *msp5* gene in wild-type (WT) and altered (Alt) colonies. A pairwise comparison was used to analyze between and within groups. Error bars indicate the s.e. and the asterisks indicate statistically significant differences between indicated groups.

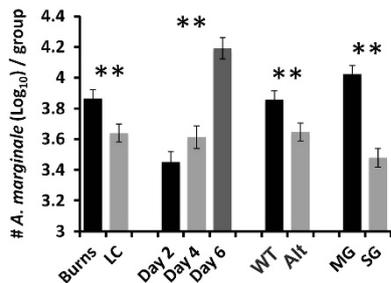


Figure 4 Global analysis of *A. marginale* infection load. *A. marginale* load in Lake Como (LC) and Burns (B) ticks is shown for day 2, day 4 and day 6 of acquisition feeding. The pathogen was quantified in the midgut (MG) and salivary glands (SG) using the single-copy *msp5* gene in wild-type (WT) and altered (Alt) colonies. A mixed-model approach was used to look at the *A. marginale* acquisition in its entirety. Error bars indicate the standard error and the double asterisk indicates statistically significant differences between indicated groups at $P < 0.01$.

Alt population as compared with the WT population in the MG on day 2 ($P < 0.046$) and day 6 ($P < 0.012$) as well as in the SG on day 6 ($P < 0.007$; Figure 3).

According to the GLM model (Figure 4), the altered microbiome affected ($P < 0.01$) *A. marginale* acquisition where Alt ticks had a lower mean (4.45×10^3 *A. marginale*) than WT ticks (7.21×10^3 *A. marginale*). This was regardless of the population and tissue type, confirming treatment differences detected in the pairwise analysis. In addition,

increased amounts of *A. marginale* within ticks occurred over time with significant differences detected between day 6 and day 2 ($P < 0.01$) and day 4 ($P < 0.01$; Figure 4). Tick MGs had higher ($P < 0.01$) mean *A. marginale* loads than SGs. However, one two-way interaction, day by organ, and one three-way interaction, population by treatment by day, were significant ($P < 0.01$). Specifically for the two-way interaction, day 2 MG was different from SG ($P < 0.05$), day 2 was different compared with day 4 ($P < 0.02$) and day 6 ($P < 0.01$) for MG, day 2 was different ($P < 0.01$) than day 6 for SG, MG was different ($P < 0.01$) from SG on day 4 and SG were different ($P < 0.01$) on day 4 compared with day 6. Therefore, *A. marginale* acquisition increased by day (Figure 4).

Quantitative analysis of *R. bellii*

The Lake Como population of ticks was colonized by an endosymbiont, *R. bellii*, which was not detected in the Burns population. The Alt treatment had a significant ($P < 0.020$) effect on *R. bellii* numbers (Figure 5a), resulting in higher levels of *R. bellii* in Alt ticks (8.40×10^7 *R. bellii* per tick) as compared with WT ticks (4.80×10^7 *R. bellii* per tick; Figure 5). These treatment differences mirror the proportional changes seen in the sequence data. Using the GLM model, tick generation ($P > 0.51$) did not result in detectable differences in *R. bellii*. However, tissue type was approaching significance ($P > 0.07$) with MG having lower levels of *R. bellii* compared with SG, similar to what was detected through sequence analysis. In addition, two two-way interactions were significant ($P < 0.01$), treatment by organ type (Figure 5b) and treatment by generation (Figure 5c). The SG from WT ticks had lower levels ($P < 0.01$) of *R. bellii* than Alt ticks. Furthermore, SG had greater numbers ($P < 0.01$) of *R. bellii* than the MG from Alt ticks. Antibiotic exposure of F1 ticks impacted *R. bellii* levels in the F2 generation with Alt ticks having greater ($P < 0.01$) numbers of *R. bellii* than WT ticks. For Alt ticks, F2 had increased ($P < 0.04$) *R. bellii* compared with F1. When coupled with the proportion data, it is clear that antibiotic exposure resulted in F2 ticks with higher levels and proportions of *R. bellii* in the Alt Lake Como microbiome as compared with the WT counterparts ($P < 0.01$; Figure 5c).

Because Lake Como ticks acquired significantly less *A. marginale* than Burns ticks, and had an endosymbiont, which was not present in Burns ticks (*R. bellii*), we assessed whether there was a correlation between the numbers of *R. bellii* in F2 ticks and the *A. marginale* infection load. A negative correlation was observed with higher levels of *R. bellii* correlating with lower levels of *A. marginale* in Alt MG and SG ($r^2 = -0.4472$, $P < 0.55$ and -0.8944 , $P < 0.10$, respectively). Significance was diminished owing to small sample size ($n = 3$ pools/correlation).

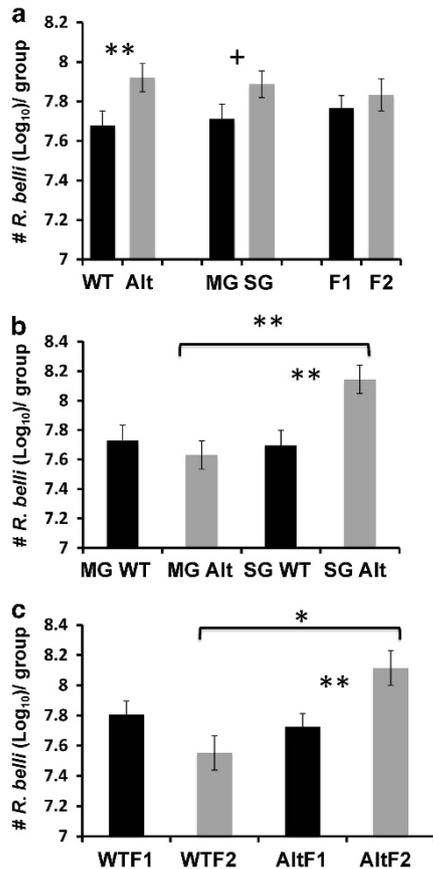


Figure 5 Comprehensive quantitation of *Rickettsia bellii* in Lake Como ticks. The amount of *R. bellii* in the midgut (MG) and salivary glands (SG) from F1 and F2 ticks is shown. (a) one-way interactions analyzed the treatment, tissue and generation effect on the quantity of *R. bellii*; (b) two-way interaction of tissue and treatment on the quantity of *R. bellii*; (c) two-way interaction of treatment and generation on the quantity of *R. bellii*. Error bars indicate the s.e. and the single asterisk indicates statistically significant differences between indicated groups at $P < 0.05$, the double asterisk indicates $P < 0.01$, whereas the plus sign indicates statistical significance at $P < 0.07$.

F. novicida acquisition

Next, we determined whether changes to the composition of *Francisella* spp. within the microbiome altered the ability of *F. novicida* to colonize the tick. For F2 nymphal ticks that fed on *F. novicida*-infected mice, the overall nymph-feeding success was 30% for Lake Como ticks and 58% for Burns ticks, averaged across treatment groups (Supplementary File 1 Supplementary Table 9). Although the sample size varied based on population and treatment, nymphs from the WT Burns population had the greatest feeding ($\chi^2(3) = 96.08$, $P < 0.001$) and molting success rate ($\chi^2(3) = 32.71$, $P < 0.001$), whereas nymphs from the Alt Lake Como population had the lowest feeding and molting success rate (Supplementary File 1 Supplementary Table 9). The prevalence of *F. novicida* infection in tick groups was 100%, except for the Lake Como WT group where infection prevalence was 86% (Supplementary File 1 Supplementary Table 9).

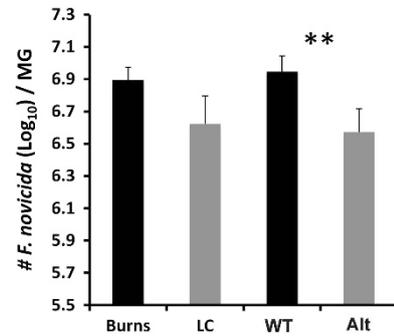


Figure 6 *F. novicida* infection load. *F. novicida* infection level in midgut (MG) of altered (Alt) versus wild-type (WT) Lake Como and Burns tick groups. Error bars indicate the s.e. and the asterisks indicate statistically significant differences between groups and the double asterisk indicates $P < 0.01$.

The lower prevalence in the latter group was due to two ticks that were likely negative owing to feeding on a mouse that developed a very low-level bacteremia (4.0×10^3 CFU ml⁻¹ blood). Figure 6 shows the *F. novicida* results of tick population and treatment. *F. novicida* CFU differed ($P < 0.02$) owing to treatment: WT ticks had more *F. novicida* (8.8×10^6 CFU) compared with Alt ticks (3.74×10^6 CFU), regardless of population. In addition, there was no difference in *F. novicida* load comparing cohorts from Lake Como and Burns ($P > 0.13$). There was not a significant treatment by population interaction ($P > 0.87$), which means that in both populations, WT ticks had increased *F. novicida* compared with Alt ticks. Although Lake Como ticks did not have a statistically significant difference in infection level between Alt and WT ticks, this is likely to be a result of high mortality of Lake Como Alt nymphs leading to a small adult sample size—there was an 82% decrease in pathogen load in Lake Como Alt ticks as compared with WT ticks. Similarly, there was a 47% decrease in pathogen load when comparing the Burns Alt cohort with WT.

Discussion

We examined the microbiomes of two tick populations from geographically distinct areas based on a previous observation of differing pathogen susceptibility to *A. marginale* (Scoles et al., 2005). The tick population from Burns was primarily colonized with *Francisella* spp. and FLE in the MG and *Arsenophonus* spp. in the SG, whereas the population from Lake Como was colonized with *Francisella* spp., FLE and *R. bellii* in the MG and *R. bellii* in the SG. In order to associate the microbiome with pathogen acquisition, we altered the microbiome by antibiotic exposure. The microbiome response to alteration varied based on location and tissue type. In the F1 Burns population, the proportion of *Arsenophonus* spp. increased and the proportion of *Francisella* spp. and FLE decreased in the MG and SG. However, in the F1 Lake Como population, *R. bellii* increased

in proportion in the MG and SG, whereas *Arsenophonus spp.* decreased in only the MG, and FLE decreased in only the SG. One generation later, F2, the microbiome was characterized again to understand if the ALT populations would restore WT phenotype. However, that was not the case, with Burns ALT population having an increased proportion in *Arsenophonus spp.* and decreased proportion of *Francisella spp.* and FLE in the MG and no compositional difference between WT and ALT in the SG. Furthermore, in F2 Lake Como, there was an increased proportion of *R. bellii* in the MG and SG, an increased proportion of *Arsenophonus spp.* in the MG, and a decreased proportion of *Francisella spp.* and FLE in both MG and SG.

One generation after microbiome alteration, the acquisition susceptibility to *A. marginale* was analyzed. Our results show that although Burns and Lake Como ticks become infected with different levels of *A. marginale*, there is not a significant difference in the infection rate, as seen previously (Scoles *et al.*, 2005). Our previous study used a less-sensitive technology (nested PCR) to determine infection rate, whereas we used ddPCR, and therefore the threshold of detection may account for the difference between studies. However, a more likely reason for the difference in results is that there was reorganization of the microbiome of Burns and Lake Como ticks over the 10-year period between sampling. Because the bacterial community was not assessed in the previous study, any changes to the microbiome of these populations between 2004 and 2012 are unknown.

In this study, we provide evidence of a negative correlation between *R. bellii*, an endosymbiont of *D. andersoni* found in the Lake Como tick population and *A. marginale*. As a population, ticks with higher levels of *R. bellii* had decreased acquisition levels of *A. marginale* as compared with ticks with lower levels of *R. bellii* (Figures 4 and 5). Although this result is preliminary and requires additional experimentation for definitive proof, previous studies have shown that *Rickettsia* endosymbionts have a negative effect on pathogenic rickettsial species in several species of ticks (Niebylski *et al.*, 1997; Macaluso *et al.*, 2002; Telford, 2009; Oliver *et al.*, 2014). These earlier studies have focused on the rickettsial interference in ovarian tissue colonization or transovarial transmission, without investigation of whether rickettsial interference occurs in additional pathogen acquisition- or transmission-associated tissues. These studies identified bacterial interference between species within the genus *Rickettsia*, and we found that such interference could occur between rickettsial species in different families. Importantly, other factors need to be considered for a complete understanding of the acquisition dynamics, such as tick genetics and complex interactions, which could affect the acquisition of *A. marginale*.

The theoretical application of rickettsial interference as a method to combat pathogen acquisition and

transmission is not a novel idea (Telford, 2009); however, it has remained within the realm of studies on human rickettsial pathogens. Our study demonstrates that this phenomenon could occur in veterinary-relevant pathogens, as seen with a decrease in *A. marginale* load (Figure 3) in ticks with higher proportion and quantity of *R. bellii*. Two possible mechanisms of rickettsial interference include a direct and indirect inhibition pathway (Figure 7). The direct mechanism entails *R. bellii* releasing a molecule that limits replication of *A. marginale*, whereas the indirect mechanism involves *R. bellii* preferentially utilizing host resources necessary for pathogen replication (Weiss and Aksoy, 2011). Regardless of pathway, bacterial inhibition appears to affect the susceptibility of ticks to *A. marginale*.

We tested the acquisition of a second pathogen, *F. novicida*, in our two populations of ticks to determine whether the microbiome affected acquisition in the same manner as for *A. marginale*. When microbiome compositions are overlaid with *F. novicida* acquisition results, our results suggest a positive relationship between the proportion of the microbiome occupied by *Francisella* endosymbionts and the infection level of *F. novicida*. This could indicate that *Francisella* endosymbionts promote a more favorable environment for the related pathogen to replication within the MG. One possible explanation is that the *Francisella* endosymbionts are causing immunosuppression of the host, which then

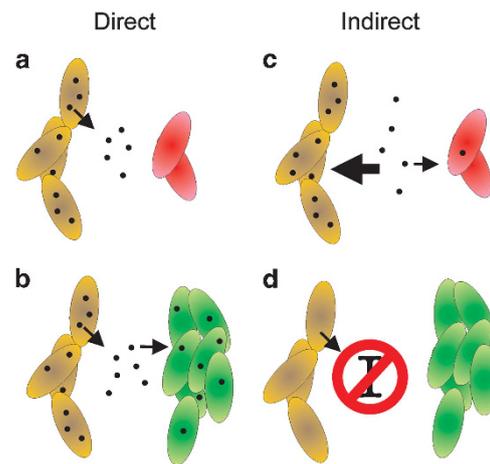


Figure 7 Mechanisms of interaction between endosymbionts and pathogens. Panels **a** and **b** show how the endosymbiont can directly affect the growth of a pathogen, whereas panels **c** and **d** show how the endosymbiont can indirectly affect the growth of a pathogen. In panel **a**, the endosymbiont secretes a compound (black dot) that has a negative effect on pathogen growth. Panel **b** shows the endosymbiont secretes a compound (black dot) that has a positive effect on pathogen growth. Panel **c** shows the endosymbiont preferentially taking up nutrients such that the pathogen cannot acquire it, thus leading to poor growth of the pathogen. Panel **d** depicts the endosymbiont affecting the immune response (symbolized by I) of the tick such that these immune factors cannot act on the pathogen leading to increased growth of the pathogen. The endosymbiont is shown in yellow in all panels. Pathogen is shown in red when it does not grow well in the presence of the endosymbiont and in green when it does grow well in the presence of the endosymbiont.

allows *F. novicida* to avoid host defenses (Figure 7). This type of symbiotic exploitation is seen in several species of mosquitoes between *Wolbachia* and *Plasmodium* (Ze'le et al., 2014), as well as between *Wolbachia* and Baculoviruses (Graham et al., 2012). In addition, activation of the Toll and Imd immune pathways in *Drosophila melanogaster* have been shown to increase the amount of *Spiroplasma* endosymbionts, which results in an increase host susceptibility to other pathogens (Herren and Lemaitre, 2011). Furthermore, *Francisella* endosymbionts may secrete a molecule, such as a nutrient, that facilitates *F. novicida* replication (Figure 7). Endosymbionts have been shown to possess the ability to produce many beneficial molecules, such as *Blochmannia* endosymbionts supplying essential amino acids or *Wigglesworthia* releasing B vitamins (Kuriwada et al., 2010). Further investigation is needed to tease apart the mechanism of interaction.

In conclusion, a disruption in the microbiome resulted in an increase in the proportion of *R. bellii* and a decrease of *Francisella* endosymbionts. These alterations demonstrated two meaningful results: a negative correlation between higher levels of *R. bellii* and *A. marginale* acquisition levels and a positive relationship between *Francisella* endosymbionts and *F. novicida* acquisition levels. These endosymbiont-pathogen interactions pose an interesting opportunity to explore our ability to decrease vector competence in *D. andersoni* by microbiome manipulation. Our results warrant further investigation into the bacterial inhibition mechanisms between *R. bellii* and *A. marginale* and between *Francisella* endosymbionts and pathogens, as well as testing these relationships for other pathogens transmitted by *D. andersoni*. We propose that different mechanisms of interaction can be at play for different pathogen-endosymbiont systems (Figure 7), which will require detailed analysis to elucidate.

Conflict of Interest

The authors declare no conflict of interest.

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