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Borrelia afzelii Infection in the Rodent Host Has Dramatic Effects on the Bacterial Microbiome of Ixodes ricinus Ticks

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1 **Title: *Borrelia* infection in rodent host has dramatic effects on the microbiome of ticks**

2

3 **Running Title: *Borrelia* changes tick microbiome**

4

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23 **Keywords:** *Borrelia*, dysbiosis, *Ixodes*, Lyme disease, microbiome, tick-borne disease, vector

24

25 **Abstract/Summary:**

26 Vector-borne diseases remain major causes of human morbidity and mortality. It is increasingly
27 recognized that the community of microbes inhabiting arthropods can strongly affect their vector
28 competence, but the role of the tick microbiome in *Borrelia* transmission – the cause of Lyme
29 disease – remains unclear. Here, we use a large-scale experiment to clarify the reciprocal
30 interactions between *Borrelia afzelii* and the microbiome of *Ixodes ricinus*, its primary vector. In
31 contrast to other reports, we find that depletion of the bacterial microbiome in larval ticks has no
32 effect on their subsequent acquisition of *B. afzelii* during blood feeding on infected mice. Rather,
33 exposure to *B. afzelii*-infected hosts drives pervasive changes to the tick microbiome, decreasing
34 overall bacterial abundance, shifting bacterial community composition, and increasing bacterial
35 diversity. These effects appear to be independent of the acquisition of *B. afzelii* by ticks,
36 suggesting they are mediated by physiological or immunological aspects of *B. afzelii* infection in
37 the rodent host. These strong effects further suggest that the dynamics of *Borrelia* infection in
38 ticks and their rodent hosts have the potential to affect other tick-associated microbes or
39 pathogens.

40

41 **Introduction:**

42 Infectious diseases vectored by arthropods impose an enormous burden on human health
43 (Jones et al. 2008, Kilpatrick and Randolph 2012, McGraw and O'Neill 2013). For successful
44 transmission, however, vector-borne pathogens must contend with both the immunological
45 defenses of the arthropod vector, and the community of other microbiota that inhabit it (Weiss
46 and Aksoy 2011, Narasimhan and Fikrig 2015, Bonnet et al. 2017, de la Fuente et al. 2017), and

47 it is now clear that endogenous microbes can shape the competence of diverse arthropod vectors
48 in acquiring and transmitting pathogens (Dong et al. 2009, Cirimotich et al. 2011, Narasimhan et
49 al. 2014, Gall et al. 2016, Abraham et al. 2017). Experimental perturbations of the microbiome
50 ('dysbiosis') via the use of antibiotics or other methods have, in various contexts, been shown to
51 either increase or decrease the susceptibility of arthropods to colonization by vector-borne
52 pathogens (Dong et al. 2009, Narasimhan et al. 2014, Abraham et al. 2017, Li et al. 2018). In
53 particular, the introduction of the intracellular bacterium *Wolbachia* into mosquito vectors
54 (McMeniman et al. 2009) can dramatically reduce mosquito competence to vector arboviruses,
55 malaria parasites, and filarial nematodes (Kambris et al. 2009, Moreira et al. 2009, Bian et al.
56 2010). These effects appear to be mediated through the innate immune system of the arthropod
57 host, rendering the mosquito less susceptible to infection by diverse pathogens (Kambris et al.
58 2009, Moreira et al. 2009, Bian et al. 2010). Similarly, *Enterobacter* bacteria in the midgut of
59 Anopheline mosquitoes produce reactive oxygen species (ROS) that can kill malaria parasites
60 (Cirimotich et al. 2011). Such direct and indirect antagonistic interactions have formed the basis
61 of increasingly sophisticated biological control strategies that include the ongoing use of
62 *Wolbachia* to control Dengue virus transmission by *Aedes* mosquitoes (McMeniman et al. 2009,
63 Hoffmann et al. 2011, Walker et al. 2011, McGraw and O'Neill 2013).

64 Hard ticks remain among the most important vectors of infectious disease in the northern
65 hemisphere, transmitting numerous pathogens that include the causative agents of Lyme
66 borreliosis ('Lyme disease'), anaplasmosis, babesiosis, and tick-borne encephalitis (Kurtenbach
67 et al. 2006, Lindquist and Vapalahti 2008, Dantas-Torres et al. 2012, Stuen et al. 2013). In
68 particular, the increasing incidence of Lyme and other tick-borne diseases in parts of Europe and
69 North America has underscored the public health risks associated with hard ticks (Ogden et al.

70 2009, Godfrey and Randolph 2011, Mysterud et al. 2016, Gasmi et al. 2017, Mysterud et al.
71 2017). Recent work on *Ixodes scapularis* has suggested that perturbations to the tick's
72 microbiome can influence tick susceptibility to *B. burgdorferi* sensu stricto (ss) and *Anaplasma*
73 *phagocytophilum* by affecting the integrity of the tick midgut (Narasimhan et al. 2014, Abraham
74 et al. 2017), although the generality and importance of these effects in the natural transmission of
75 these pathogens are unclear. It is likewise unclear if and when specific members of the tick
76 microbiota can interfere with *Borrelia* colonization of the tick, or if *Borrelia* impacts the tick
77 microbiota in ways that might affect the dynamics of other tick-borne diseases.

78 Here, we use a large-scale experiment with tick offspring derived from wild-collected
79 gravid tick mothers to investigate reciprocal interactions between *Borrelia afzelii*, an endemic
80 cause of Lyme disease in Europe (van Duijvendijk et al. 2015), and the microbiome of *Ixodes*
81 *ricinus*, its primary vector. We found that disrupting the microbiome of larval *I. ricinus* ticks by
82 bleaching egg casings has profound yet transient effects on the tick microbiota, but no effect on
83 subsequent colonization by *B. afzelii* when ticks feed on *B. afzelii*-infected hosts. In contrast,
84 feeding on *B. afzelii*-infected mice has profound and pervasive effects on the composition and
85 diversity of the tick microbiome, which are largely independent of prior microbiome disruption.
86 This suggests that *B. afzelii* infection dynamics within rodent hosts have strong potential to
87 sculpt the tick microbiota, with unclear consequences for the transmission of other tick-borne
88 pathogens. Negative interactions between *B. afzelii* and other members of the tick microbiome
89 might provide insights into the biological control of tick-borne diseases.

90

91 **Materials and Methods:**

92 **Borrelia, ticks, and mice:** We used *Borrelia afzelii* isolate NE4049, which was obtained
93 from an *I. ricinus* nymph at a field site near Neuchâtel, Switzerland. This strain has multi-locus
94 sequence type ST679, *ospC* major group (oMG) allele A10, and strain ID number 1887 in the
95 *Borrelia* MLST database. We used isolate NE4049 because we have previously shown that it is
96 highly infectious for both mice and ticks (Jacquet et al. 2016, Belli et al. 2017, Jacquet et al.
97 2017). Pathogen-free, female *Mus musculus* BALB/c ByJ mice were used as the vertebrate
98 reservoir host. To infect mice via tick bite, we used *Ixodes ricinus* nymphs infected with isolate
99 NE4049 that had been generated in a previous study (Belli et al. 2017) and that came from our
100 laboratory colony of *Borrelia*-free *I. ricinus* ticks. For these *I. ricinus* nymphs, the percentage of
101 nymphs infected with *B. afzelii* ranged between 80.0% and 100.0%. Uninfected control nymphs
102 were obtained from our laboratory colony of *Borrelia*-free *I. ricinus* ticks.

103 **Experiment:** The experimental design of the study is shown in **Figure 1**. Engorged adult
104 female *I. ricinus* ticks were collected from wild roe deer captured in the Sylve d'Argenson forest
105 near Chizé, France. The female ticks were allowed to lay their eggs in the laboratory. Four weeks
106 after deposition, each of the 10 clutches of eggs was split into two batches. One batch was rinsed
107 with 10% bleach while the other batch was rinsed with water (**Figure 1**). The rinsed eggs were
108 allowed to hatch into larvae under non-sterile conditions. To test whether the bleach treatment
109 had reduced the microbiota in the larval ticks, a group of ~400 larvae was frozen for each of the
110 20 batches at six weeks after hatching.

111 The remaining larvae for each of the 20 batches were split into two groups of ~100
112 larvae. For each of the 20 batches of eggs (10 tick families x 2 egg washing treatments), one
113 group of larvae was fed on an uninfected control mouse (n = 20 control mice) whereas the other
114 group of larvae was fed on a *B. afzelii*-infected mouse (n = 20 infected mice; **Figure 1**). These

115 mice had been infected with *B. afzelii* (or not) via nymphal tick bite (see below for details). The
116 resultant engorged larvae were placed in individual Eppendorf tubes and allowed to moult into
117 nymphs. Four weeks after the larva-to-nymph moult, 10 nymphs were randomly selected from
118 each mouse and were frozen at -80°C (**Figure 1**). In summary, we froze 400 nymphs (10
119 families*2 egg washing treatments* 2 mice infection statuses*10 nymphs/mouse).

120 **Experimental infection of mice:** For the main experiment, 40 BALB/c mice were
121 randomly assigned to either the control group or the infection group. Each mouse in the control
122 group (n = 20) was infested with 5 uninfected *I. ricinus* nymphs, whereas each mouse in the
123 infected group (n = 20) was infested with 5 *B. afzelii*-infected *I. ricinus* nymphs. Each of the 40
124 mice had been infested with 5 nymphs, so that each mouse had similar immune experience with
125 ticks. Five weeks after the nymphal challenge, an ear tissue biopsy and a blood sample were
126 taken from each of the 40 mice. The ear tissue biopsy was tested for the presence of *B. afzelii*
127 infection using qPCR. The blood sample was tested for *Borrelia*-specific antibodies using a
128 commercial Lyme disease ELISA. These tests confirmed that the 20 mice in the infected group
129 were infected with *B. afzelii*, whereas the 20 mice in the control group were uninfected. These 40
130 mice were used to feed the larval ticks (see above and in **Figure 1**).

131 **Molecular methods for larval ticks:** The 20 groups of larval ticks that had been frozen
132 were split into two sub-groups with ~200 larval ticks per sub-group. Half of these sub-groups
133 were washed with ethanol prior to DNA extraction and the other sub-groups were not washed.
134 DNA extraction of the 40 sub-groups of larval ticks was done using a QIAGEN kit following the
135 manufacturer's instructions. The DNA of each sub-group was eluted into 100 μl of distilled
136 water. The DNA concentration was measured for each of the 40 sub-groups using a Nanodrop.
137 For qPCR, the DNA concentration of each sub-group was adjusted to 5 $\text{ng}/\mu\text{l}$. Two qPCR assays

138 were performed independently for each DNA extraction: tick *calreticulin* and bacterial *16S*
139 *rRNA*. Each qPCR assay contained 3 µl of template for a total of 15 ng of DNA.

140 **Molecular methods for nymphal ticks:** For each mouse, the 10 nymphs were split into
141 two groups of 5 nymphs. Ticks in one group were washed with ethanol prior to DNA extraction
142 and ticks in the other group were not washed. DNA extraction of whole ticks was done using a
143 QIAGEN kit following the manufacturer's instructions. The DNA of each tick was eluted into 65
144 µl of distilled water. The DNA concentration was measured for each of the 400 nymphs using a
145 Nanodrop. For qPCR, the DNA concentration of each tick was adjusted to 5 ng/µl. Three qPCR
146 assays were performed independently for each DNA extraction: tick *calreticulin*, bacterial *16S*
147 *rRNA*, and *Borrelia flagellin* (see electronic supplementary material (ESM) for details). Each
148 qPCR assay contained 3 µl of template for a total of 15 ng of DNA.

149 **Illumina Library Preparation and Sequencing of the *16S rRNA* gene:** Of the 400
150 nymphs for which we had quantified bacterial load using the *16S rRNA* gene qPCR assay,
151 Illumina sequencing was performed for 360 nymphs. Sample preparation consisted of two PCR
152 reactions. In the first reaction we amplified a 464 bp fragment of the V3-V4 region of the *16s*
153 *rRNA* gene using primers Bakt_341F (5'CCTACGGGNGGCWGCAG-3') and Bakt_805R
154 (5'GACTACNVGGGTATCTAATCC-3') (Herlemann et al. 2011), designed with Illumina
155 adapters. Reactions were performed in a final volume of 50 µl using 2.5 U of HotStar HiFidelity
156 DNA polymerase (Qiagen,Germany), 2.5 µl of 10 µM primers, 10 µl of 15 µM dNTP mix, with
157 a thermal cycle with a denaturation step of 95°C for 5 min, 45 cycles of 94°C for 15 sec, 51°C
158 for 45 seconds, and 72°C for 45 seconds, with a final elongation step at 72°C for 7 minutes.
159 Amplicons were purified with the Wizard SV Gel and PCR Clean-Up system (Promega
160 Switzerland).

161 The second PCR incorporated the sample barcodes. Reactions were performed in a final
162 volume of 25 µl using 1.25 U of HotStar HiFidelity DNA polymerase, 1 µl of 10 µM primers, 5
163 µl of 15 µM dNTP mix. The thermocycler had a denaturation step of 95°C for 5 min, 12 cycles
164 of 95°C for 30 sec, 55°C for 30 seconds, and 72°C for 30 seconds, with a final elongation step of
165 72°C for 5 minutes, and amplicons purified as above. The 360 purified amplicons were pooled in
166 equimolar concentration using a Qubit 2.0 fluorometer (Invitrogen) and sequenced by
167 Microsynth (Balgach, Switzerland) using an Illumina MiSeq v2 with 250 bp paired end output,
168 followed by adaptor and quality trimming.

169

170 **STATISTICAL METHODS:**

171 **Analysis of the bacterial load in the larvae:** For each sub-group of larval ticks, we
172 divided the *16S rRNA* gene copy number by the *calreticulin* gene copy number. These *16S rRNA*
173 to *calreticulin* ratios were log₁₀-transformed to improve the normality of the data. The log₁₀-
174 transformed *16S rRNA* to *calreticulin* ratios were analyzed using linear mixed effects models
175 (LMMs). Fixed factors included egg washing treatment (2 levels: water and bleach), larval tick
176 washing treatment (2 levels: none and ethanol), and their interactions. Random factors included
177 tick family. We used R/Bioconductor (v 3.4.2. or above) for analyses, including the *lme4*,
178 *complexHeatmap*, *vegan*, and *phyloseq* packages (McMurdie and Holmes 2013, Bates et al.
179 2015, Gu et al. 2016, Oksanen et al. 2019).

180 **Data selection of the nymphs:** Of the 370 DNA extractions, 14 were not included in the
181 analysis because their DNA concentrations were too low. After adjustment of the DNA
182 concentration, the remaining 356 DNA extractions had a DNA concentration that ranged

183 between 3.33 and 5.00 ng/ μ l so that the 3 μ l of DNA template contained between 10.0 and 15.0
184 ng of DNA.

185 **Analysis of the bacterial load in the nymphs:** For each tick, we divided the *16S rRNA*
186 gene copy number by the *calreticulin* gene copy number. These ratios were log10-transformed to
187 normalize the data. The log10-transformed *16S rRNA* to *calreticulin* ratios were analysed using
188 LMMs. Fixed factors included egg washing treatment (2 levels: water and bleach), *B. afzelii*
189 infection status of the mouse (2 levels: uninfected control, infected), nymphal tick washing
190 treatment (2 levels: none and ethanol), and their interactions. Random factors included tick
191 family and mouse identity nested inside tick family.

192 **Analysis of the *B. afzelii* infection prevalence in the nymphs:** These analyses were
193 restricted to the subset of nymphs that had fed as larvae on the *B. afzelii*-infected mice. We used
194 a proportion test to determine whether the microbiome reduction of the egg washing treatment
195 influenced the susceptibility of the nymphs to acquire *B. afzelii* infection during the larval blood
196 meal. The nymphal infection status (0 = uninfected, 1 = infected) was also analysed using
197 generalized linear mixed effects models (GLMMs) with binomial errors. Fixed factors included
198 egg washing treatment, nymphal tick washing treatment, and their interaction. Random factors
199 included tick family and mouse identity nested inside tick family.

200 **Analysis of the *B. afzelii* spirochete load in the nymphs:** This analysis was restricted to
201 the subset of nymphs that were infected with *B. afzelii*. For each tick, we divided the *B. afzelii*
202 *flagellin* gene copy number by the tick *calreticulin* gene copy number. These ratios were log10-
203 transformed to normalize the data. The log10-transformed *flagellin* to *calreticulin* ratios were
204 analysed using LMMs. Fixed factors included egg washing treatment, nymphal tick washing

205 treatment, and their interaction. Random factors included tick family and mouse identity nested
206 inside tick family.

207 **Analysis of 16S rRNA amplicons:** Because of limited overlap between the 250 bp read
208 ends, we elected to use an OTU picking strategy that did not require first assembling paired end
209 reads into contigs. We generated OTU tables using the CD-HIT-OTU-Miseq workflow (Li and
210 Chang 2017) packaged with CD-HIT v 4.6.8. Forward and reverse read lengths were specified at
211 200 and 150 bp and clustered against the SILVA 132 99% OTU release (Quast et al. 2013),
212 otherwise using default parameters that included using Trimmomatic for read trimming (Bolger
213 et al. 2014). This identified 10,454 OTUs, although the vast majority of reads (93.6%) recruited
214 to the 100 most abundant OTUs. It also assigned taxonomy to only 926 OTUs (< 10%): for more
215 robust taxonomic assignments, we applied Metaxa2 v2.2 (Bengtsson-Palme et al. 2015) to the
216 representative sequences for each OTU identified by CD-HIT using default parameters and the
217 included reference database in Metaxa2. This identified 7,550 OTUs as bacterial and provided
218 taxonomies that were largely congruent with CD-HIT assignments, where evaluable; subsequent
219 analyses were restricted to this bacterial OTU set.

220 The OTU table and taxonomy were imported to R (>v. 3.4.2), and analyzed using the
221 *phyloseq*, *vegan*, and *DeSeq2* packages (as detailed in Results). Linear mixed models for
222 univariable outcomes were implemented using the *lme4* package with a family as a random
223 effect, or nesting mouse host within tick family. In analyses using *DeSeq2* and *db-RDA*, family
224 was included as a fixed factor (*DeSeq2*) or a conditioning variable (*db-RDA*). We evaluated the
225 reliability of our replicated sequencing/analysis approach on the same nymphal samples via the
226 variance explained by sample in *db-RDA*, and the intraclass correlation coefficient for Shannon
227 diversity. For statistical analyses requiring tick infection status, we imputed missing infection

228 status for one *Borrelia*-exposed nymph as infected, as that was the most common state of
229 exposed nymphs.

230

231 **Results:**

232 *Egg bleaching profoundly disrupts the microbiome of tick larvae*

233 Egg bleaching had strong effects on the abundance of bacteria associated with tick larvae,
234 as shown by a 27.5-fold reduction in the relative *I6S rRNA* gene (hereafter *I6S*) copy number
235 (scored via qPCR) in larvae hatching from bleached versus unbleached eggs (N = 29 evaluable
236 samples; linear mixed model (LMM) controlling for tick family; $P < 10^{-6}$; \log_{10} ratio of
237 *I6S/calreticulin* 0.24 versus 6.61, respectively; **Figure 2A**). This reduction was evident six
238 weeks after treatment, demonstrating a profound effect of bleaching on the larval tick
239 microbiome. In contrast, washing larvae with ethanol prior to DNA extraction, which is expected
240 to reduce the external microbiota (but see Binetruy et al. 2019), did not have a significant effect
241 on the *I6S* copy number (LMM; $P = 0.12$; **Figure 2A**). Taken together, these observations
242 suggest that the egg bleaching-induced reduction of *I6S* copy number was driven by an increase
243 in the relative abundance of the internal microbiota of the larval ticks at the expense of the
244 external microbiota.

245 Multivariate analysis of the larval tick microbiome using db-RDA revealed that egg
246 bleaching led to a clear shift in the *I6S* community, whereas there was no discernible effect of
247 washing the larvae prior to DNA extraction (db-RDA; based on Bray-Curtis dissimilarities of
248 \log_{10} OTU abundances, stratified by tick family. Permutation tests; $P < 0.001$ and 0.727,
249 respectively; **Figure 2B**). Consistent with our expectations, the most significantly
250 (proportionally) enriched taxon due to bleaching was in the order Rickettsiales, which we further

251 manually annotated to *Candidatus* Midichloria mitochondrii, an endosymbiont of *I. ricinus* that
252 we expect would be unaffected by external bleaching (**Figure 2C**; $P_{\text{adj}} < 10^{-6}$; shown are the \log_{10}
253 read counts per thousand mapped reads). In contrast, egg bleaching significantly reduced the
254 relative abundance of Pseudomonas OTU 23 in the resultant larvae, which suggests that this
255 bacterium is found on the surface of the eggshell (**Figure 2C**; $P_{\text{adj}} < 10^{-3}$). Other significant
256 effects associated with bleaching included an increase in *Methylobacterium*, a taxon that has
257 been implicated as a potential contaminant of laboratory reagents (Salter et al. 2014); these are
258 consistent with the strong reduction in *16S* abundance we observed via qPCR. No OTUs changed
259 significantly from washing (all $P_{\text{adj}} > 0.05$). In sum, egg bleaching dramatically decreased *16S*
260 copy number and shifted the microbial community composition in larvae measured at 6 weeks
261 after treatment. Egg bleaching probably reduced the relative abundance of bacteria associated
262 with the egg surface and thereby increased the relative abundance of endosymbiont bacteria. The
263 lack of an observed effect of washing the larvae—on both microbial abundance and microbial
264 diversity—suggests that external microbiota are a minor component of the *16S* diversity in the
265 lab-hatched tick larvae.

266

267 *Manipulation of the larval microbiome has no effect on the acquisition of B. afzelii, and the*
268 *microbiome largely recovers in the unfed nymphs*

269 We screened nymphs for *B. afzelii* infection via qPCR, and found no evidence that
270 disruption of the larval microbiome affected either the percentage of ticks that acquired the
271 infection while feeding (with infection prevalences of 68.2% and 73.5% in unbleached and
272 bleached groups respectively; binomial GLMM; $P = 0.44$, $N = 183$, **Figure 3A**) or *B. afzelii* copy
273 number in the nymphs that became infected (LMM; $P = 0.958$, $N = 130$; **Figure 3B**). Thus,

274 although the egg bleaching treatment was highly effective at reducing the microbiome in larvae,
275 it did not affect the ability of *B. afzelii* to colonize ticks during or following the larval blood
276 meal.

277 Although the egg bleaching treatment strongly reduced the *I6S* copy number in the
278 larvae, the bacterial community largely recovered after these larvae had taken a blood meal and
279 molted into nymphs, as there was no effect of the egg bleaching on *I6S* copy number in nymphs
280 (LMM; $P = 0.272$, $N = 356$, **Figure 3C**). Intriguingly, in contrast to the non-significant effect of
281 ethanol washing on *I6S* copy number that we observed in larvae, there was a clear reduction
282 with both washing and *B. afzelii* exposure. The *I6S* copy number was lowest in ticks that were
283 both washed and *B. afzelii*-exposed (LRT for interaction; $P < 10^{-5}$), indicating that feeding on *B.*
284 *afzelii*-infected mice reduced the internal bacterial load in nymphs; we investigate this hypothesis
285 in more detail below.

286

287 *Host infection with B. afzelii has pervasive effects on the microbiome of I. ricinus nymphs*

288 To extend our analysis of larval ticks to the nymphal stage, we used *I6S* amplicon
289 sequencing to profile replicate nymphs from each treatment alongside the larval samples (above).
290 We again restricted these analyses to the 40 most abundant OTUs. As expected from prior
291 reports (Heise et al. 2010, Zolnik et al. 2016), there was a clear shift in the tick-associated
292 bacterial community from larval to nymphal ticks (**Figure 4A**). There was, further, a striking
293 effect of *B. afzelii* infection in the mouse on the tick microbiota as quantified in the nymphal
294 stage, with pronounced differences between ticks fed on *B. afzelii*-infected versus control mice
295 (db-RDA; $P < 0.001$; **Figure 4B** shows unsupervised principal coordinates analysis (PCoA)). We
296 also found that egg bleaching and ethanol washing prior to DNA extraction had modest but

297 significant effects on microbiome composition in nymphs ($P < 0.001$ and $P = 0.014$, respectively;
298 **Figure 4B**), consistent with our finding that washing the nymphs decreased *16S* copy number.
299 While sequencing recovered OTUs annotated as *Borrelia*, the most abundant *Borrelia*-annotated
300 OTU was ranked 81st in overall abundance and accounted for $<0.05\%$ of total sequence reads.
301 Thus, a direct contribution of sequenced *Borrelia 16S* amplicons in infected ticks does not
302 explain these strong patterns.

303 Microbiome α -diversity (measured as Shannon entropy) in nymphs modestly increased as
304 a result of both microbiome disruption via egg bleaching (LMM; $P = 0.035$; $N = 305$. **Figure**
305 **5A**) and from *B. afzelii* infection ($P = 0.0025$; **Figure 5B**) or exposure ($P = 0.0007$; competing
306 multivariable model; **Figure 5B**). Coupled with the decrease in *16S* copy number observed when
307 feeding on *B. afzelii*-infected mice, these diversity effects appeared to be mediated through
308 disproportionately negative impacts on abundant OTUs (e.g. *Stenotrophomonas*) leading to
309 increased community evenness. As mentioned, these trends were evident when considering the
310 infection status of the tick itself, or that of the mouse on which they were fed (**Figure 5C**).
311 Visual comparison between control nymphs, uninfected nymphs, and infected nymphs
312 demonstrated that it was feeding on an infected mouse rather than acquiring *B. afzelii* infection
313 that was most important for determining the nymphal microbiome (**Figure 5C**). This was
314 supported by comparing competing models with Akaike's Information Criterion (AIC), which
315 found that feeding on an infected mouse was a much stronger predictor of both α -diversity
316 ($\Delta AIC \sim 6$) and the multivariate bacterial community (**Figure 5A**; $\Delta AIC \sim 14$ in models using
317 first PCoA axis as response variable) than acquisition of *B. afzelii* by the tick (**Figure 5**),
318 suggesting that the effects we observe are more likely to be caused by physiological or
319 immunological characteristics of infected mice rather than the direct effects of *B. afzelii* infection

320 in the ticks. Similarly, examining the OTU frequencies in bleached and *B. afzelii*-exposed ticks
321 showed that these net effects were driven by proportional reductions in the dominant OTUs, with
322 concomitant increases in less frequent OTUs (e.g. **Figure 4**). Collectively, these results are
323 consistent with the strong effects of mouse *B. afzelii* infection status we observe on *16S* copy
324 number and suggest that these are specifically mediated by disproportionate negative effects on
325 abundant bacterial OTUs.

326

327 *Microbial correlates of Borrelia exposure*

328 To characterize the recurrent shifts in the tick microbiome associated with *B. afzelii*
329 exposure, we used negative binomial models implemented in *phyloseq/DESeq2*. In line with the
330 global diversity shifts we observed, feeding on *B. afzelii*-infected mice led to significant changes
331 in the relative frequencies of many OTUs (with 19/40 significant at $P_{\text{adj}} < 0.05$; Figure 5). This
332 was most evident in large decreases in the frequency of multiple *Stenotrophomonas* OTUs and
333 other Gammaproteobacteria (**Figure 6**). As a result, there appeared to be a degree of taxonomic
334 dependence in microbial responses to *B. afzelii* exposure, with Betaproteobacteria generally
335 significantly increasing in frequency and Gammaproteobacteria decreasing (Fisher's exact test, P
336 = 0.004; **Figure 6**). Our analysis also revealed that several nymph OTUs that increased in
337 frequency in response to tick bleaching, including two *Burkholderia* OTUs (Betaproteobacteria;
338 $P_{\text{adj}} < 0.01$; **Figure 6**) and a *Bradyrhizobium* OTU (Alphaproteobacteria; $P_{\text{adj}} < 0.01$; **Figure 6**),
339 whereas no OTUs significantly (proportionally) decreased. Consistent with our expectations,
340 ethanol washing prior to DNA extraction increased the relative abundance of the endosymbiotic
341 *Candidatus* Midichloria mitochondrii (annotated as order *Rickettsiales*, Alphaproteobacteria) as

342 well as *Spiroplasma* (Mollicutes) by 2.5 and 1.7-fold respectively ($P_{\text{adj}} < 0.01$), corroborating an
343 enriching effect of washing on the internal tick microbiota.

344

345 **Discussion:**

346 Here, we used a highly replicated experiment on wild-collected tick families to examine
347 the reciprocal interactions between the endogenous tick microbiota and *B.afzelii*. We used egg
348 bleaching to radically disrupt the microbiome in larval ticks but found no evidence that this
349 affected the subsequent susceptibility of ticks to infection with *B. afzelii*. Rather, this work
350 revealed striking effects of feeding on *B. afzelii*-infected mice on the tick microbiome; these
351 effects superseded those of actually acquiring an infection, as judged by comparing competing
352 statistical models, and the resultant partitioning of samples in microbial community space (e.g.

353 **Figure 4A, 5C).**

354 A growing number of studies have investigated whether dysbiosing ticks influences their
355 susceptibility to acquiring tick-borne pathogens (Narasimhan et al. 2014, Gall et al. 2016,
356 Abraham et al. 2017, Li et al. 2018). In some systems, microbiome disruption makes tick species
357 more susceptible to infection with tick-borne pathogens (Abraham et al. 2017, Li et al. 2018),
358 whereas other systems found the opposite effect (Narasimhan et al. 2014, Gall et al. 2016). In the
359 present study, we found no evidence that microbiome disruption influenced *B. afzelii* infection
360 rates or the *B. afzelii* spirochete load in infected ticks. In contrast, a previous study found that
361 dysbiosed *I. scapularis* larvae were less likely to acquire *B. burgdorferi* ss and contained lower
362 bacterial loads compared to control larvae (Narasimhan et al. 2014). These two studies differed
363 in a number of factors including the *Borrelia* species, the *Ixodes* tick species, and the method of
364 dysbiosis. An important aspect of the present study is that we investigated whether dysbiosis of

365 the eggs influenced the infection status of the nymphs, which is the stage that is actually critical
366 for the transmission of Lyme disease in nature (Diuk-Wasser et al. 2012).

367 Despite the fact that bleaching the eggs was highly effective at reducing the bacterial
368 microbiome in the resultant larvae, this method of ‘dysbiosis’ did not have a meaningful impact
369 on *B. afzelii* transmission. While the presence of additional bacteria to those uncovered here
370 could influence these patterns, we found little influence of tick family on the recovered bacterial
371 communities, outside of potentially vertically transmitted bacteria such as *Spiroplasma*,
372 suggesting the microbial community of *I. ricinus* is largely homogenous at the scale studied here.
373 Our method of dysbiosis further highlights the neglected importance of maternal transmission of
374 gut symbionts in ticks. In arthropods, gut symbionts are typically vertically transmitted by
375 superficial bacterial contamination of eggs (egg smearing) (Salem et al. 2015). Our egg washing
376 with bleach has removed such maternally inherited gut symbionts and this impacted the
377 microbial communities hosted by larvae. Our study shows that egg smearing is a key mechanism
378 for colonization of ticks by their associated microbes.

379 *B. afzelii* infection reduced the microbial abundance (in the ethanol-washed nymphs) and
380 changed the microbial community in the unfed nymphs. Notably, we observed changes in OTU
381 relative abundance to be, at least in part, taxon-specific with decreases in Gammaproteobacteria
382 and increases in Betaproteobacteria (**Figure 6**). The observation that mouse infection status was
383 more important than tick infection status suggests that the blood physiology at the time of the
384 larval blood meal was critical for structuring the subsequent nymph microbiome. Metabolomic
385 studies of mouse serum samples have shown that *B. burgdorferi* ss infection changes the blood
386 concentration of amino acids, energy metabolites, and aromatic compounds (Glader et al. 2019),
387 which could influence the development of the tick microbiome. Infection with *B. burgdorferi* sl

388 stimulates the host immune system, which could also exert collateral damage on the tick
389 microbiome (Wooten and Weis 2001, LaRocca and Benach 2008, Kraiczy 2016, Tracy and
390 Baumgarth 2017). For example, elevated levels of complement, cytokines, leukocytes, and
391 reactive oxygen species in the blood (Seller et al. 1995, Kang et al. 1997, Schwanz et al. 2011,
392 Kraiczy 2016) may interact inside the tick to have negative effects on the midgut microbiome. In
393 summary, our study suggests that the physiological and immunological changes associated with
394 infection in the vertebrate host have important consequences for the microbiome of feeding ticks.

395

396 **Data and Code accessibility:** Raw sequencing reads will be deposited at NCBI (accession
397 pending). Scripts to reproduce analysis and figures from OTU tables are available at
398 github.com/onecarbon/tickdysbiosis.

399

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405

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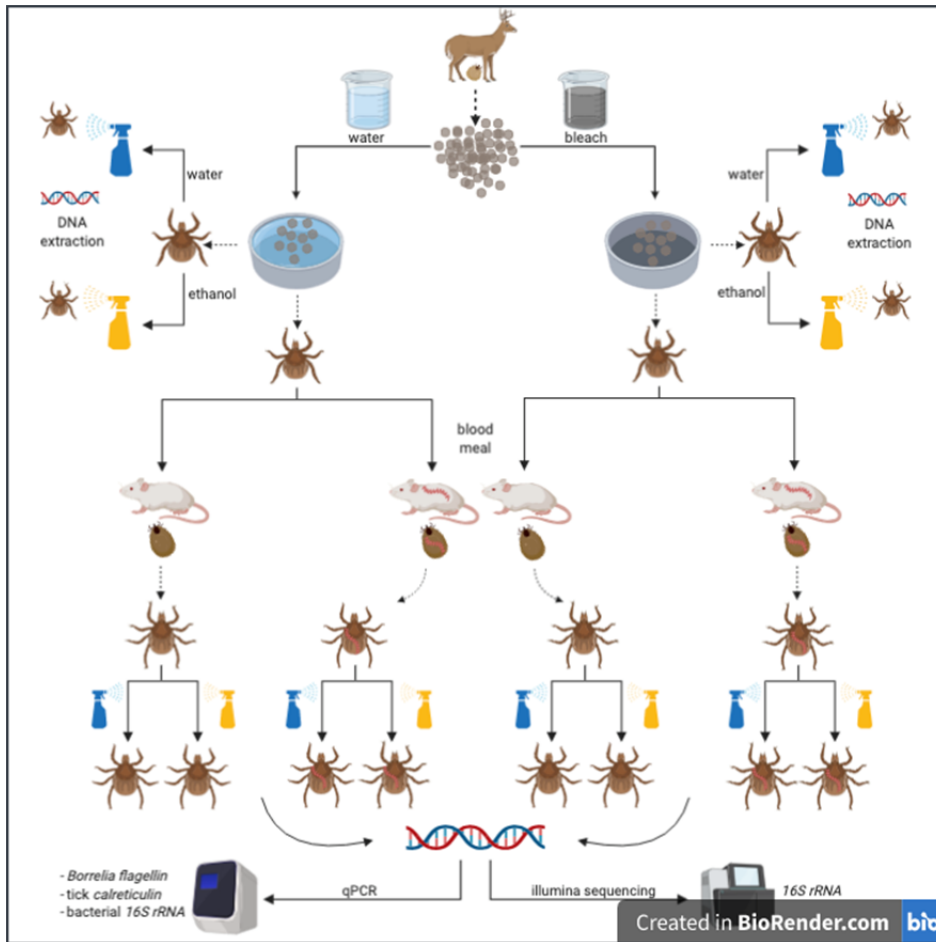
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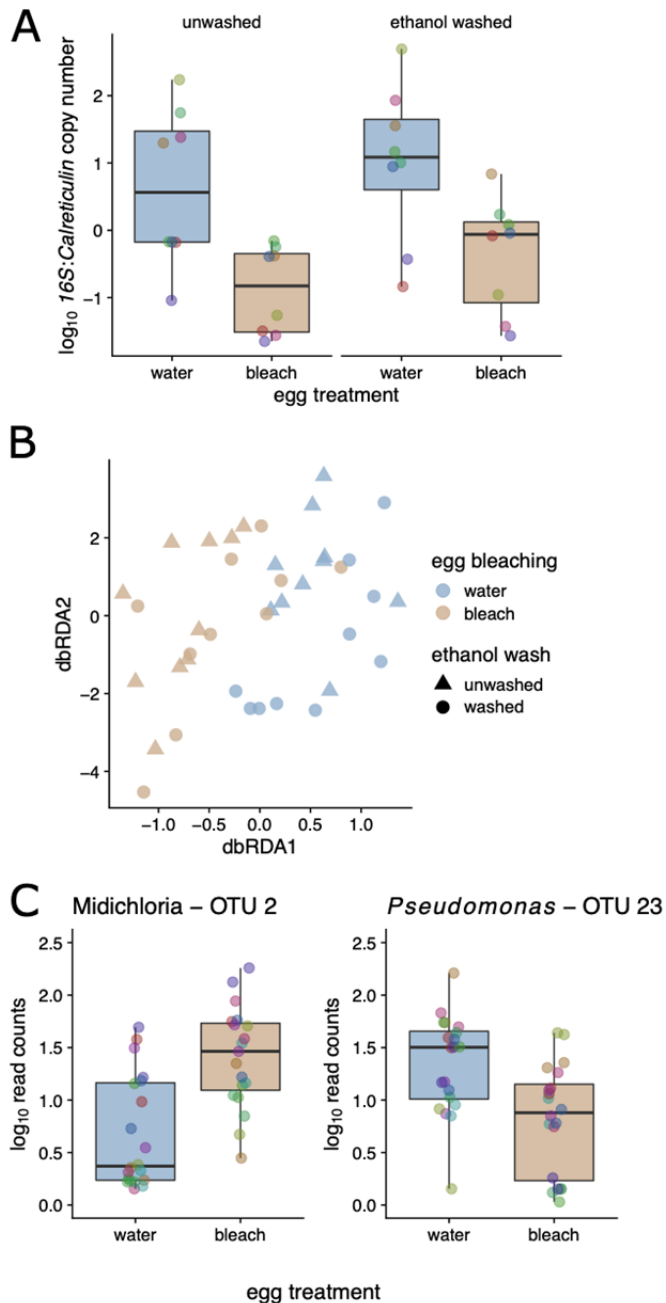


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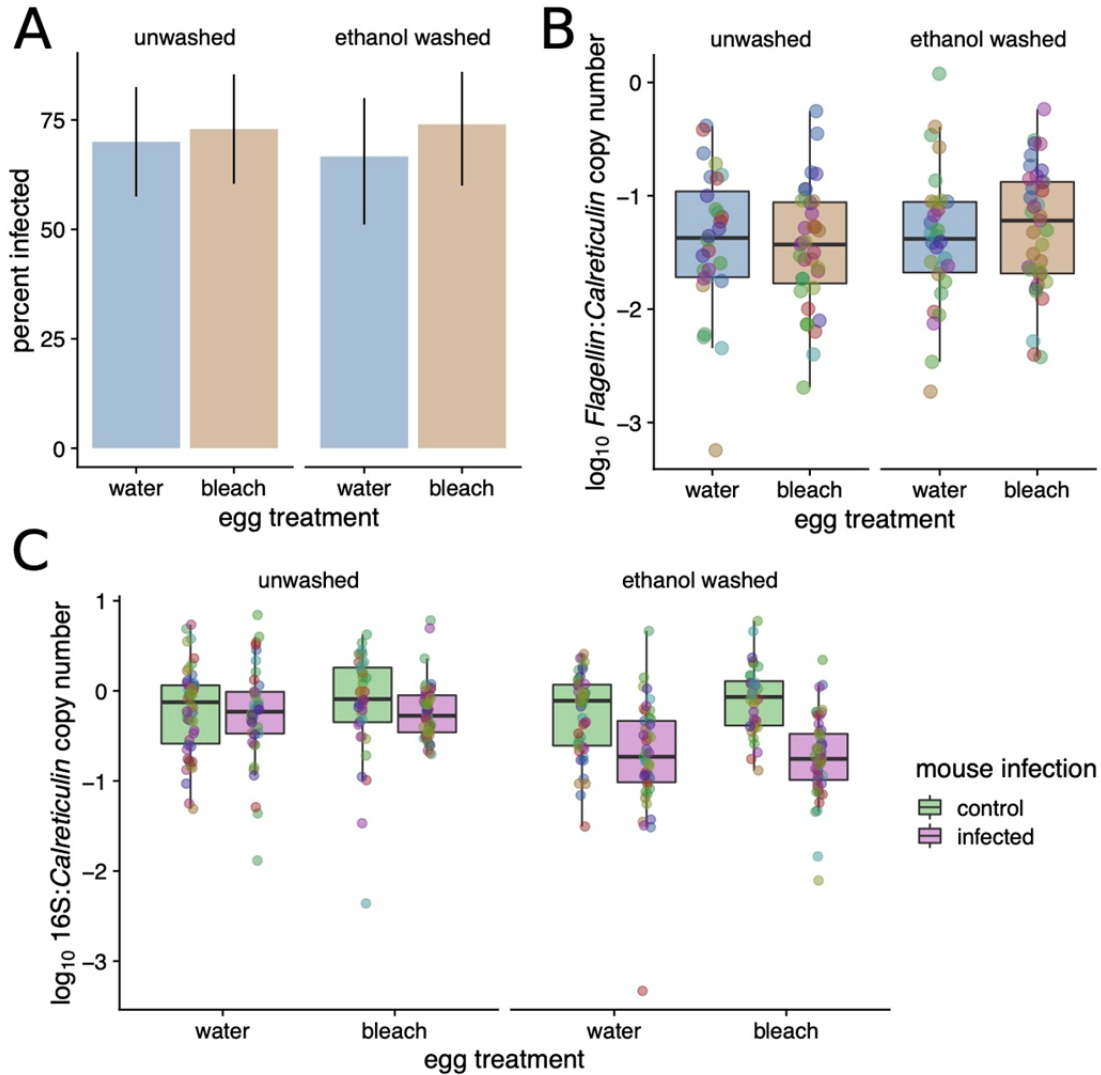
578 **Figure 1.** Experimental design. Engorged female *I. ricinus* ticks (n = 10) were collected from roe
579 deer captured in the Chizé forest, France and laid their eggs in the laboratory. Each of the 10 egg
580 batches was split into two batches and rinsed with either 10% bleach (n = 10 batches) or distilled
581 water (n = 10 batches) and hatched into larvae. To determine whether the egg bleaching
582 treatment reduced the microbiome, a subset of larvae was tested using qPCR and Illumina
583 sequencing of the bacterial *16S rRNA* gene. Larvae for each of the 20 batches were split into two
584 groups of ~100 larvae. For each of the 20 batches of eggs (10 tick families x 2 egg washing
585 treatments), one group of larvae was fed on an uninfected control mouse (n = 20 control mice)
586 whereas the other group of larvae was fed on a *B. afzelii*-infected mouse (n = 20 infected mice).
587 Engorged larvae were placed in individual Eppendorf tubes to moult into nymphs. Four weeks
588 after the moult, 10 nymphs were randomly selected from each of the 40 mice and frozen at –
589 80°C (n = 400 nymphs). These nymphs were tested for *B. afzelii* infection using qPCR and for
590 their bacterial load and microbiome using qPCR and Illumina sequencing of the bacterial *16S*
591 *rRNA* gene.

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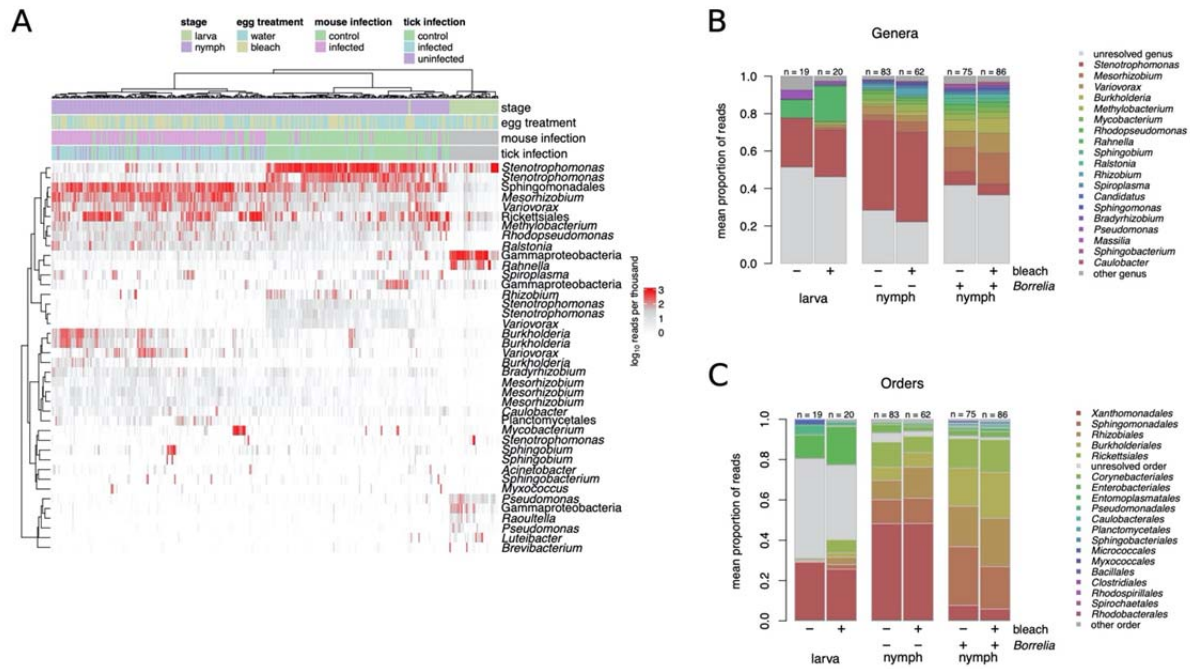


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 595 **Figure 2. Bleaching of egg casings profoundly decreases the abundance of microbiota in**
 596 **larval ticks.** Pooled *Ixodes scapularis* larvae were quantified six weeks after egg bleaching. **A.**
 597 Egg bleaching led to a ~27.5-fold reduction in the relative copy number of the 16S rRNA in
 598 larvae (vs tick *calreticulin*; $P < 0.001$), irrespective of ethanol washing prior to DNA extraction
 599 ($P > 0.05$). **B.** Egg bleaching, but not washing with ethanol prior to DNA extraction, led to
 600 significant shifts in the bacterial community, as measured by 16S amplicon sequencing and
 601 dbRDA ($P < 0.001$ and $P > 0.05$, respectively). **C.** The most enriched taxon in response to egg
 602 bleaching was *Candidatus* *Midichloria mitochondrii* ($P_{adj} < 0.001$); in contrast the most depleted
 603 was a *Pseudomonas* OUT (the Y-axis has units of number of read counts per thousand mapped

604 reads). Colored points in boxplots represent individual data points (pooled larval samples,
 605 colored by the tick mother; ‘family’; N = 10).
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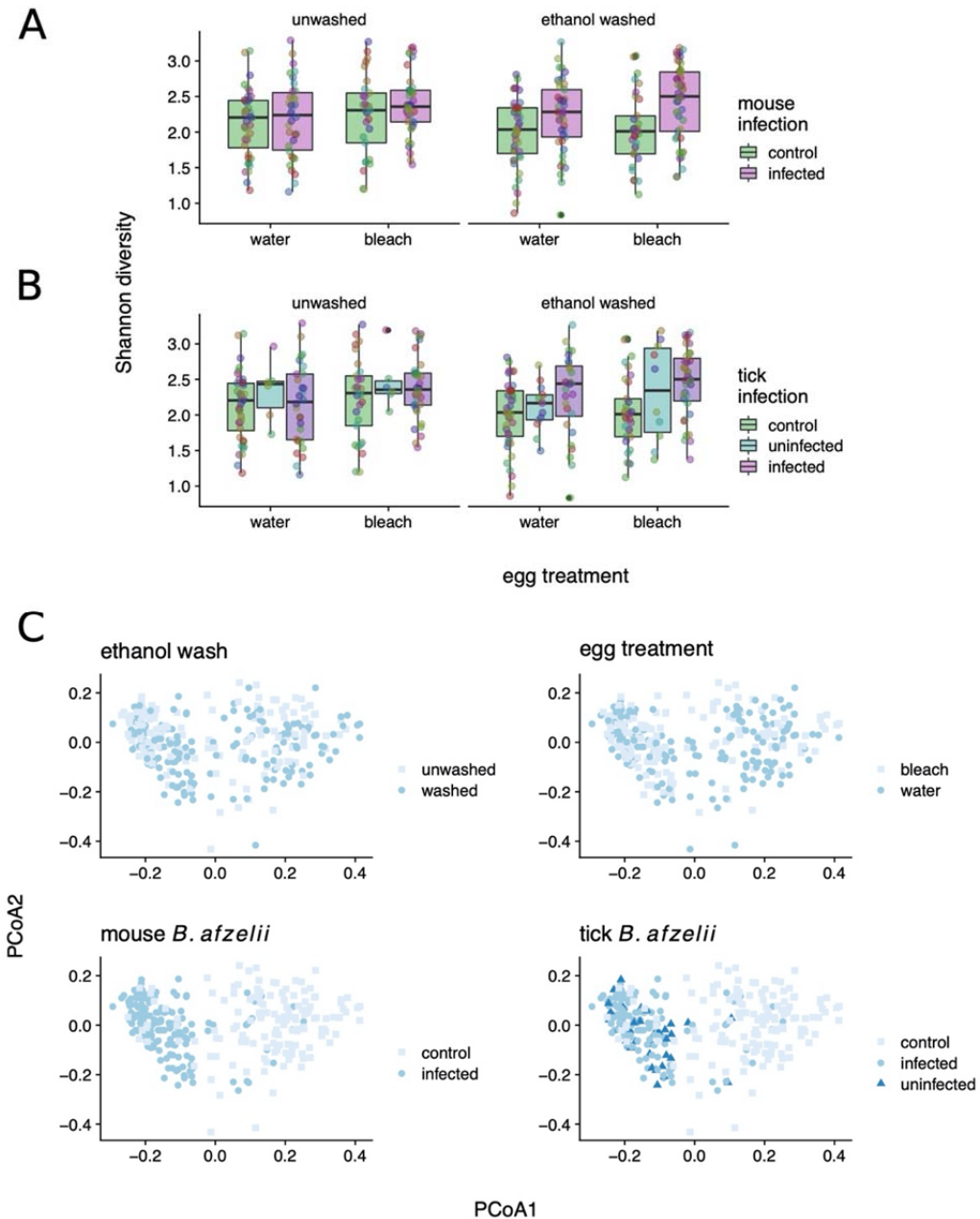


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 610 **Figure 3. ‘Dysbiosing’ larval ticks does not affect infection success by *B. afzelii*.** Neither **A.**
 611 the percentage of nymphs that acquired *B. afzelii* during their larval blood meal nor **B.** the
 612 inferred *B. afzelii* load in these nymphs were affected by prior microbiome disruption (all $P \gg$
 613 0.05). **C.** Feeding on *B. afzelii*-infected mice decreased the bacterial load in *I. ricinus* nymphs;
 614 this effect was more visible in the nymphs that were washed prior to DNA extraction compared
 615 to the unwashed nymphs.



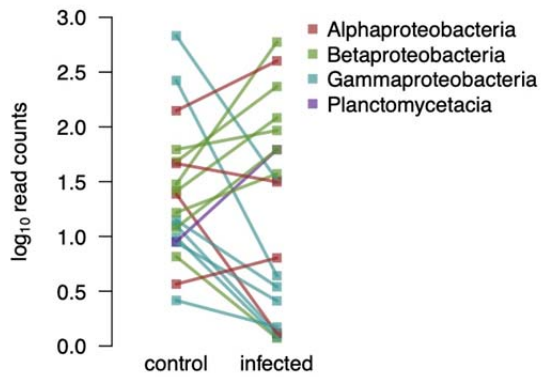
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617 **Figure 4. Life history stage and *B. afzelii* exposure affect tick microbiome.** **A.** Heatmap of
 618 number of reads assigned ($\log_{10}(x+1)$ per thousand) for top 40 OTUs across all samples in the
 619 experiment. Highest taxonomy reliably assigned by Metaxa2 is shown. Dendrograms are based
 620 on hierarchical clustering of Bray-Curtis dissimilarities using Ward's method. **B.** Composition of
 621 treatment groups and life histories, with top 40 OTUs aggregated (as mean of samples per group)
 622 at the genus level. **C.** As above, at the order level.



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624 **Figure 5. Egg bleaching and rinsing and *B. afzelii* exposure increase the diversity of the 16S**
 625 **microbiome of *I ricinus* nymphs.** Shannon diversity increases in both **A.** *B. afzelii* exposed
 626 ticks and **B.** *B. afzellii* infected ticks ($P < 0.01$). Akaike's Information Criterion (AIC) shows
 627 exposure to be a stronger predictor of Shannon diversity than infection ($\Delta\text{AIC} \sim 6$) in linear
 628 mixed models. Points represent individual tick nymphs colored by family of origin. **C.** Principal
 629 Coordinates Analysis of nymphal 16S counts, colored by experimental factors; *B afzelii* exposure
 630 best stratifies groups on PCoA 1 ($\Delta\text{AIC} \sim 11$ vs infection).
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Figure 6. Proportional abundance of taxa that changed significantly in *I. ricinus* nymphs (19/40 at $P_{adj} < 0.05$) in response to feeding on *B. afzelii* infected mice, shown at the level of class. There is significant taxonomic dependence (Fisher's exact test $P = 0.004$) of taxonomic response.

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Supplemental Material:

Data S0: Description of molecular methods

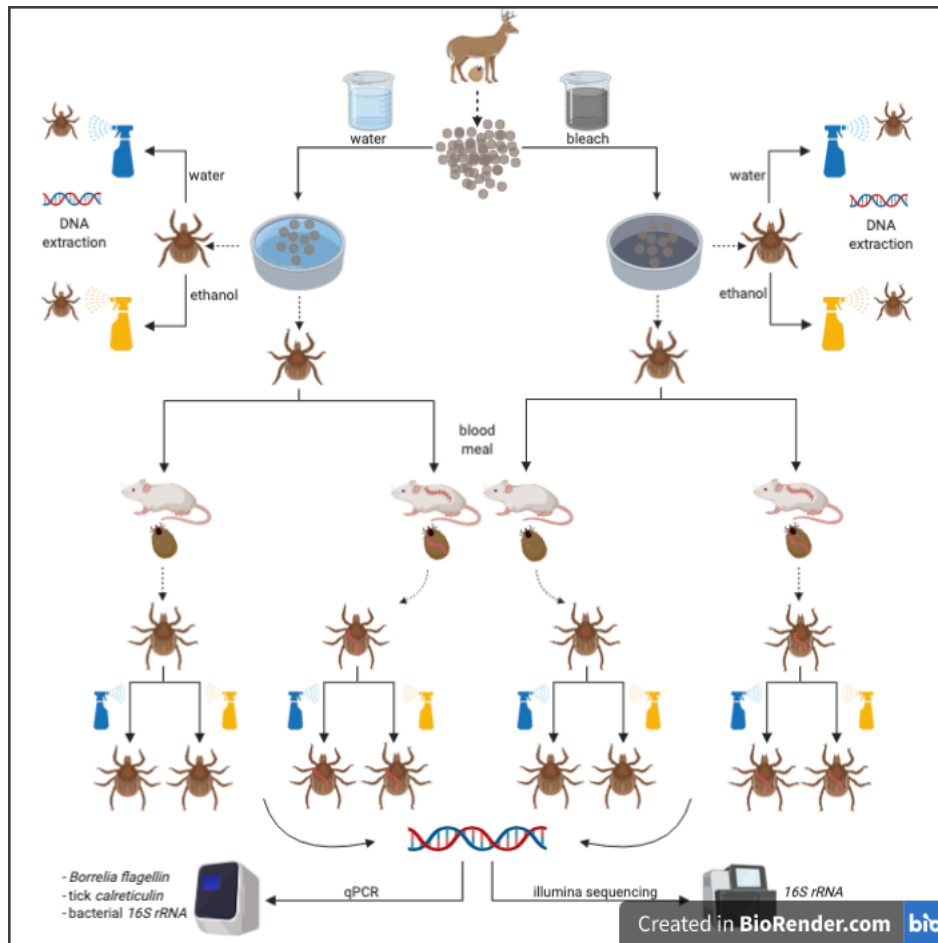
Data S1: Metadata

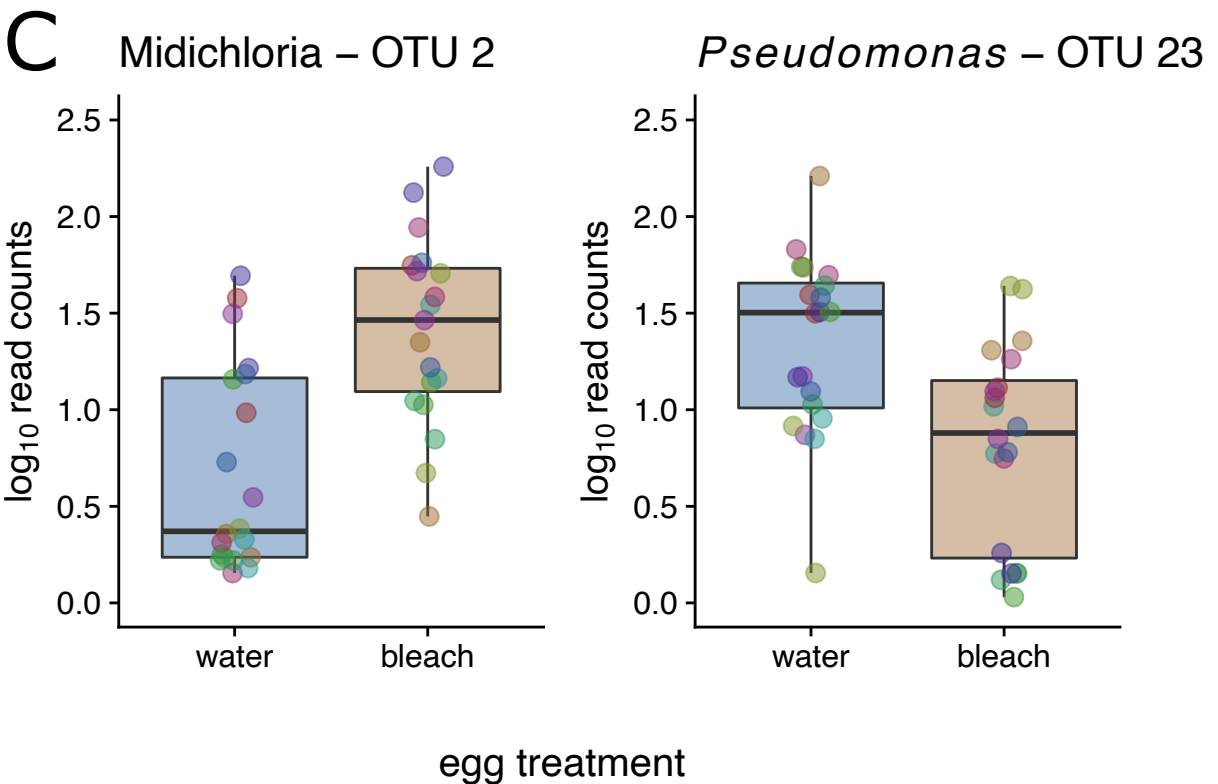
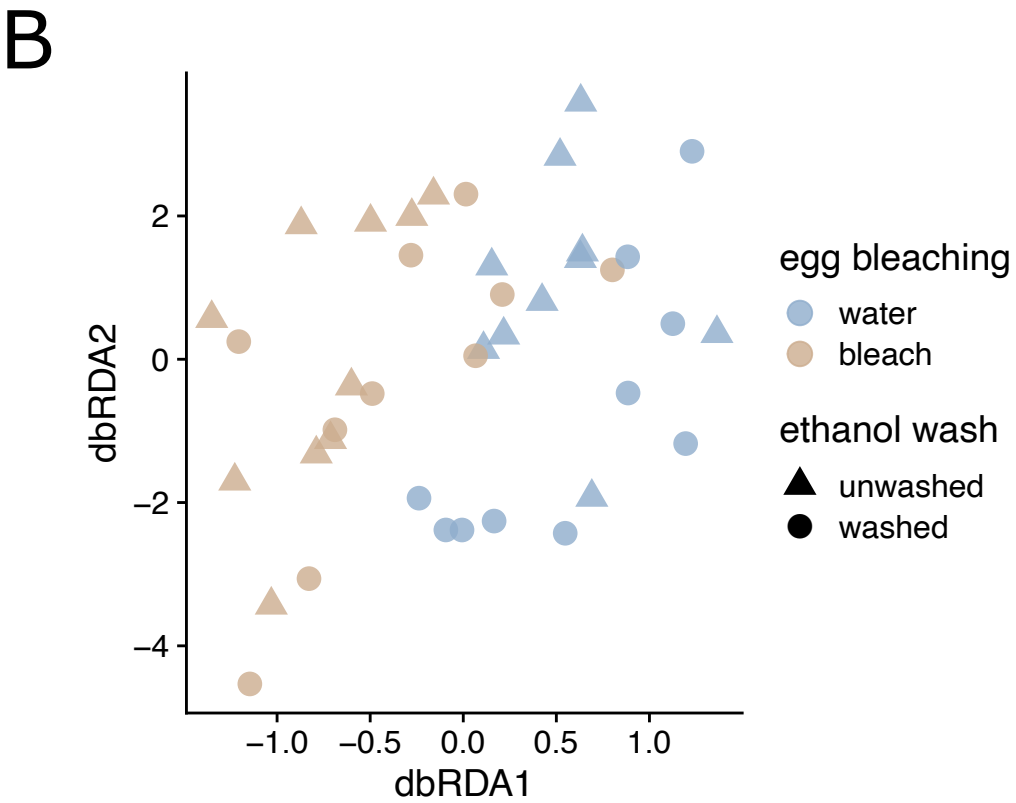
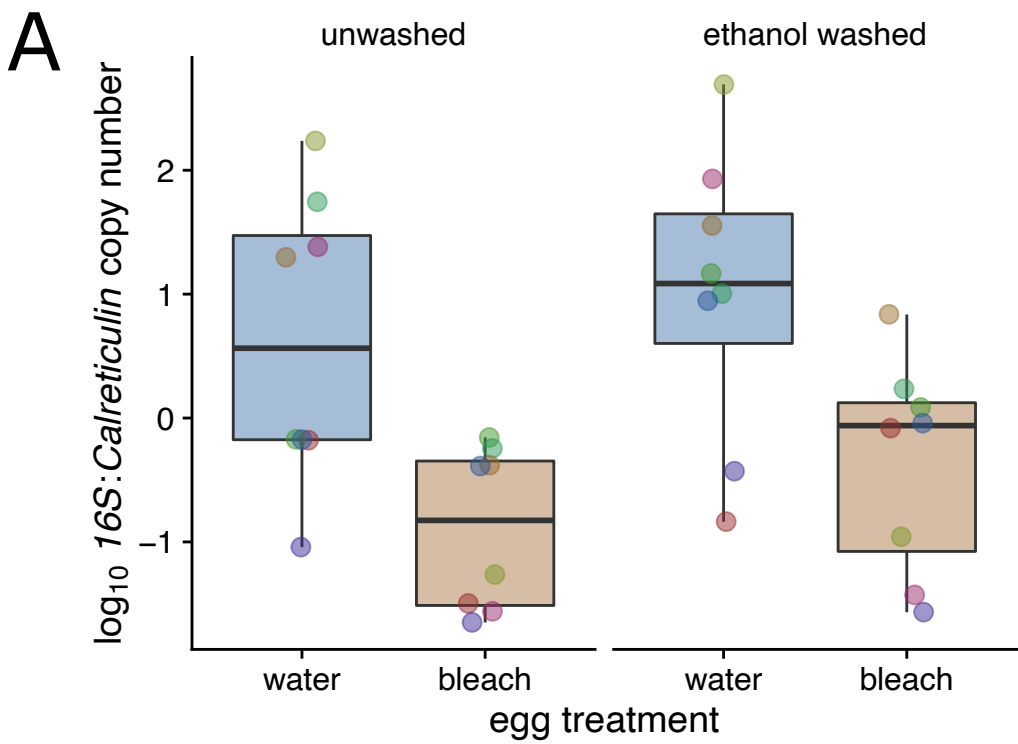
Data S2: Metadata

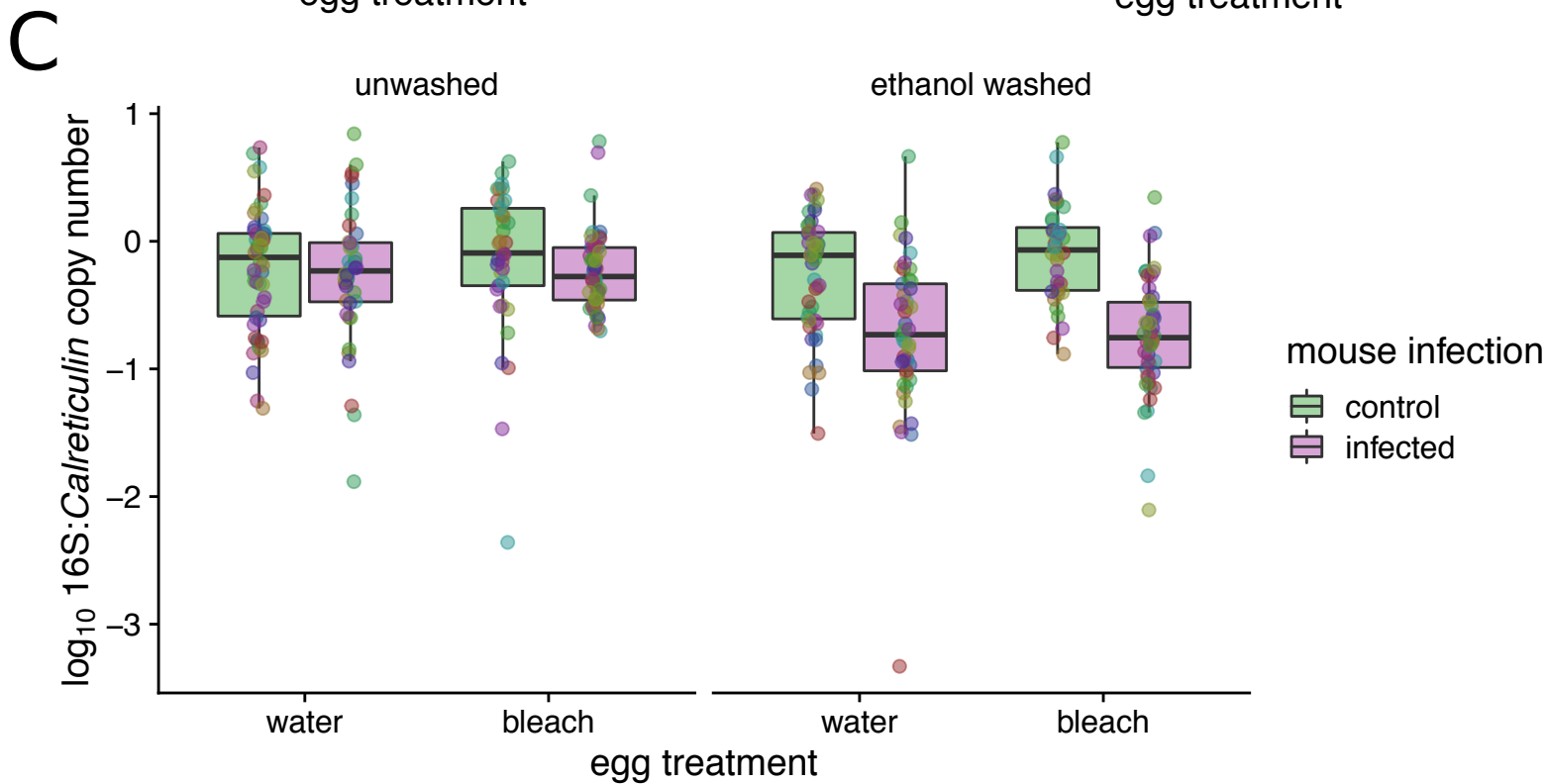
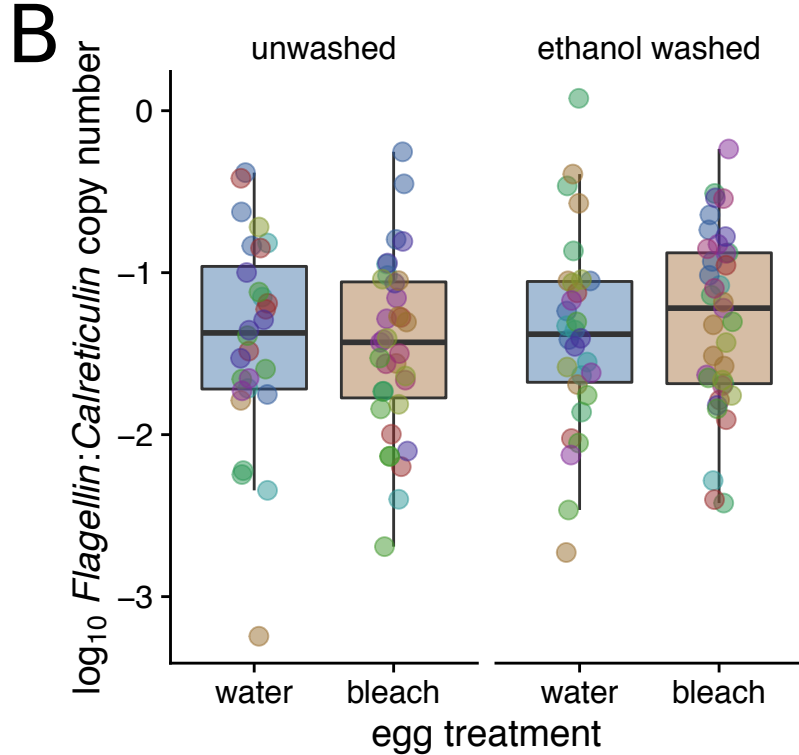
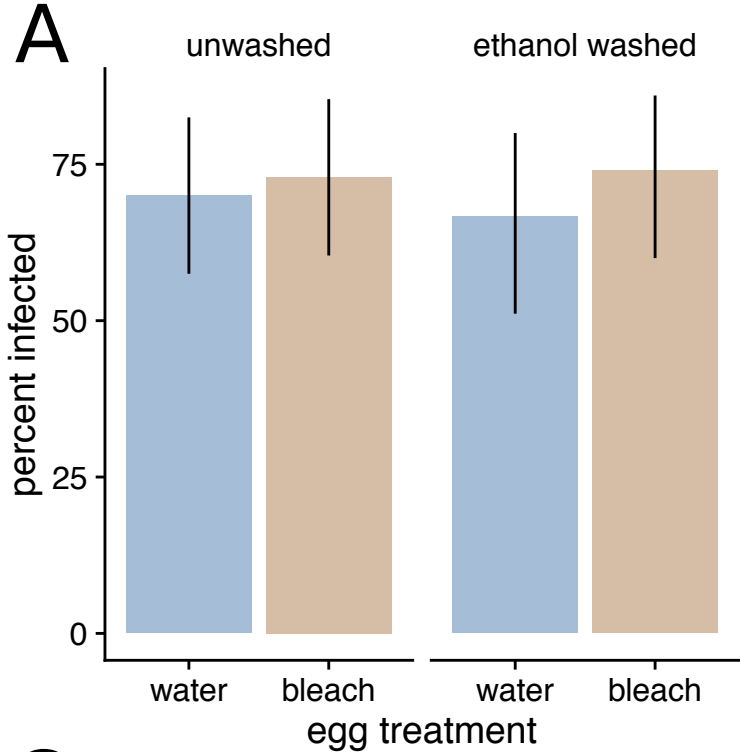
Data S3: OTU table produced by CD-HIT-OTU_MiSEQ.

Data S4: OTU clusters produced by CD-HIT-OTU_MiSEQ.

Data S5: OTI taxonomy assigned by Metaxa2.







A

stage
 larva (green)
 nymph (purple)

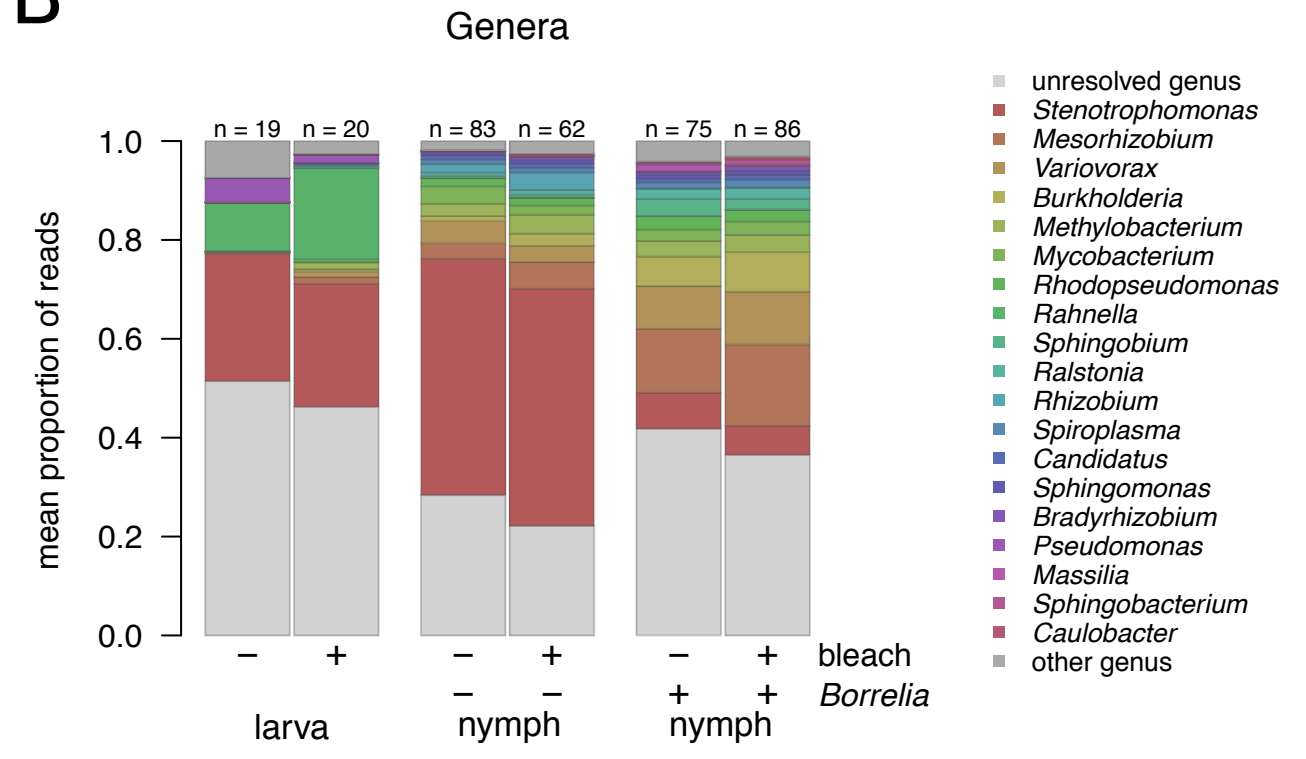
egg treatment
 water (cyan)
 bleach (yellow)

mouse infection
 control (light green)
 infected (pink)

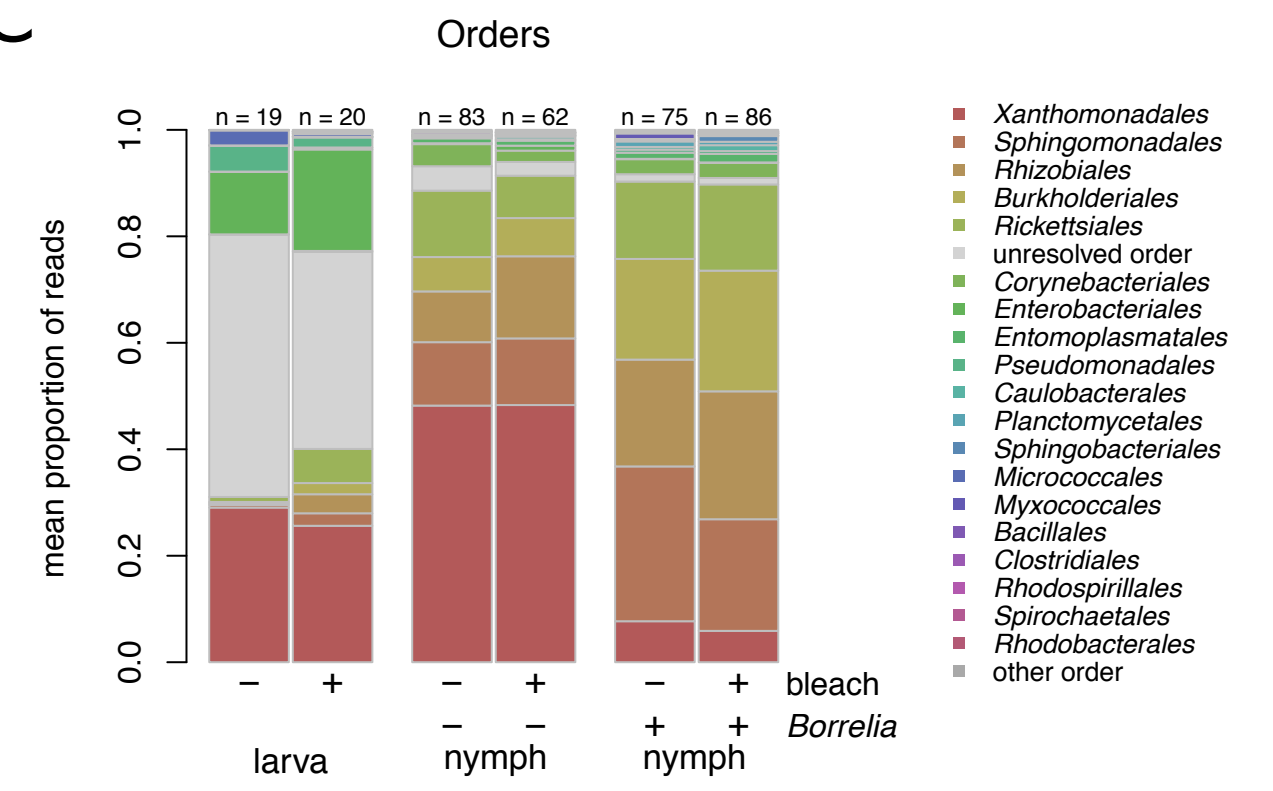
tick infection
 control (light green)
 infected (cyan)
 uninfected (purple)

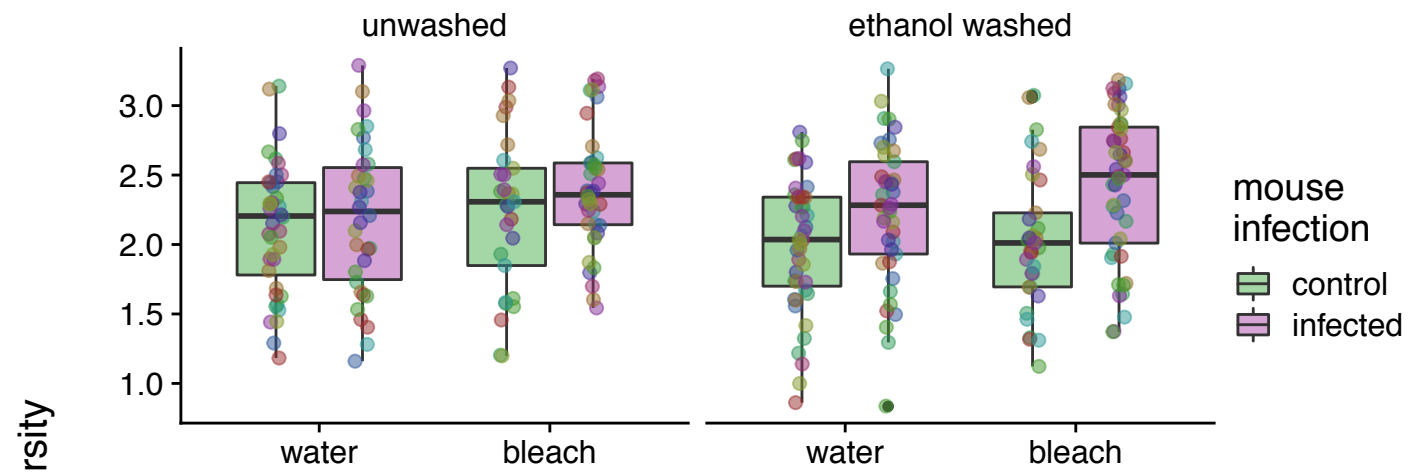
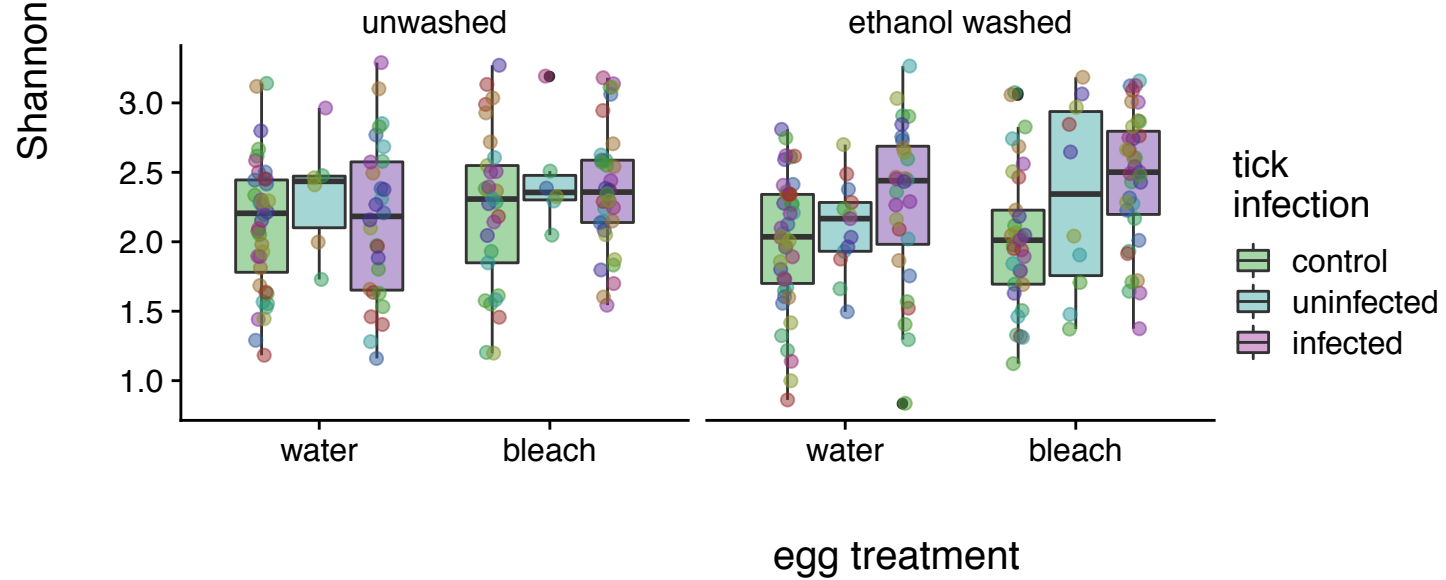


B

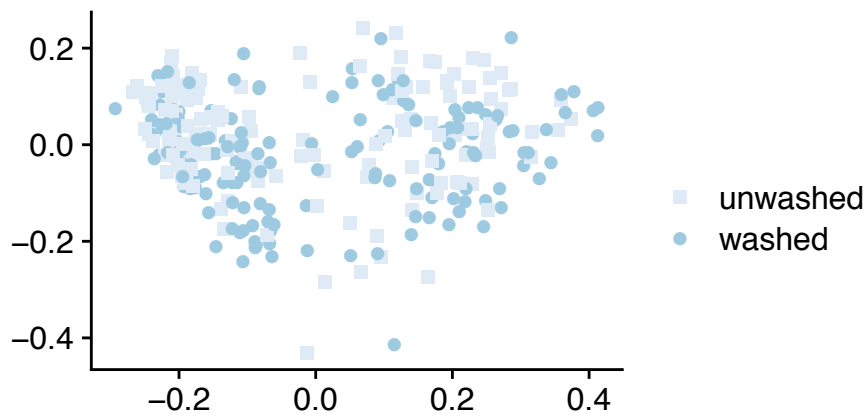


C

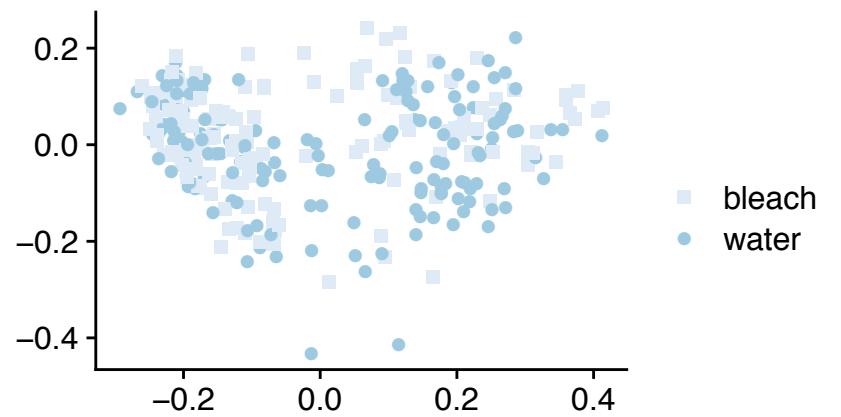
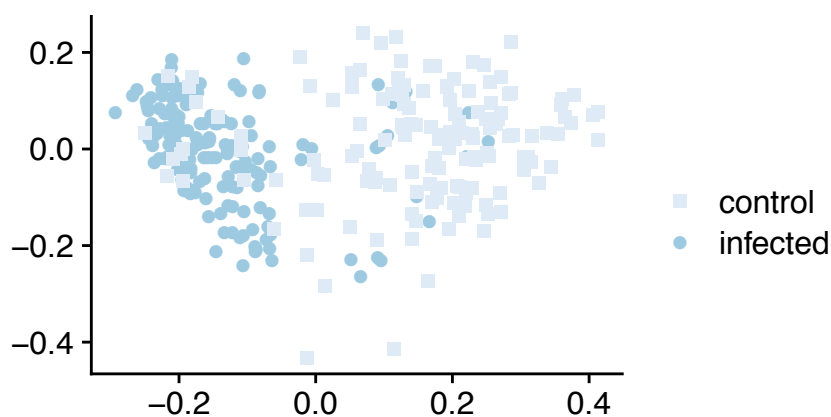
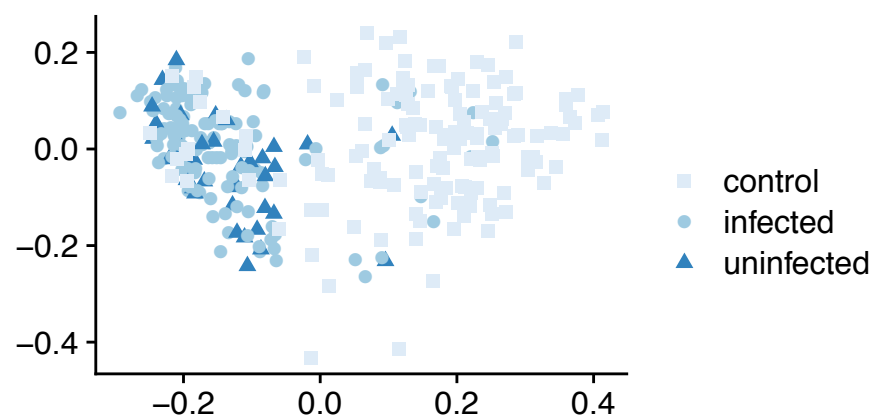


A**B****C**

ethanol wash



egg treatment

mouse *B. afzelii*tick *B. afzelii*

PCoA1

