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

2

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

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23 **Abstract:**

24 **Background:** Vector-borne diseases remain major causes of human morbidity and mortality. It
25 is increasingly recognized that the community of microbes inhabiting arthropods can strongly
26 affect their vector competence, but the role of the tick microbiome in *Borrelia* transmission – the
27 cause of Lyme disease – remains unclear.

28

29 **Results:** Here, we use a large-scale experiment to clarify the reciprocal interactions between
30 *Borrelia afzelii* and the microbiome of *Ixodes ricinus*, its primary vector. In contrast to other
31 reports, we find that depletion of the bacterial microbiome in larval ticks has no effect on their
32 subsequent acquisition of *B. afzelii* during blood feeding on infected mice. Rather, exposure to *B.*
33 *afzelii*-infected hosts drives pervasive changes to the tick microbiome, decreasing overall
34 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.

38

39 **Conclusions:** Manipulation of the microbiome of *I. ricinus* larvae had no effect on their ability
40 to acquire *B. afzelii*. In contrast, *B. afzelii* infection in the mouse had dramatic effects on the
41 composition of the gut microbiome in *I. ricinus* nymphs. Our study demonstrates that vector-
42 borne infections in the vertebrate host shape the microbiome of the arthropod vector.

43

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44 **Background**

45 Infectious diseases vectored by arthropods impose an enormous burden on human health
46 [1-3]. For successful transmission, however, vector-borne pathogens must contend with both the
47 immunological defenses of the arthropod vector, and the community of other microbiota that
48 inhabit it [4-7], and it is now clear that endogenous microbes can shape the competence of
49 diverse arthropod vectors in acquiring and transmitting pathogens [8-12]. Experimental
50 perturbations of the microbiome ('dysbiosis') via the use of antibiotics or other methods have, in
51 various contexts, been shown to either increase or decrease the susceptibility of arthropods to
52 colonization by vector-borne pathogens [8, 10, 12, 13]. In particular, the introduction of the
53 intracellular bacterium *Wolbachia* into mosquito vectors [14] can dramatically reduce mosquito
54 competence to vector arboviruses, malaria parasites, and filarial nematodes (Kambris et al. 2009,
55 Moreira et al. 2009, Bian et al. 2010). These effects appear to be mediated through the innate
56 immune system of the arthropod host, rendering the mosquito less susceptible to infection by
57 diverse pathogens [15-17]. Similarly, *Enterobacter* bacteria in the midgut of Anopheline
58 mosquitoes produce reactive oxygen species that can kill malaria parasites [9]. Such direct and
59 indirect antagonistic interactions have formed the basis of increasingly sophisticated biological
60 control strategies that include the ongoing use of *Wolbachia* to control Dengue virus
61 transmission by *Aedes* mosquitoes [3, 14, 18, 19].

62 Hard ticks remain among the most important vectors of infectious disease in the northern
63 hemisphere, transmitting numerous pathogens that include the causative agents of Lyme
64 borreliosis ('Lyme disease'), anaplasmosis, babesiosis, and tick-borne encephalitis [20-23]. In
65 particular, the increasing incidence of Lyme and other tick-borne diseases in parts of Europe and
66 North America has underscored the public health risks associated with hard ticks [24-28]. Recent

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67 work on *Ixodes scapularis* has suggested that perturbations to the tick's microbiome can
68 influence tick susceptibility to *B. burgdorferi* sensu stricto (ss) and *Anaplasma phagocytophilum*
69 by affecting the integrity of the tick midgut [8, 12], although the generality and importance of
70 these effects in the natural transmission of these pathogens are unclear. It is likewise unclear if
71 and when specific members of the tick microbiota can interfere with *Borrelia* colonization of the
72 tick, or if *Borrelia* impacts the tick microbiota in ways that might affect the dynamics of other
73 tick-borne diseases.

74 Here, we use a large-scale experiment with tick offspring derived from wild-collected
75 gravid tick mothers to investigate reciprocal interactions between *Borrelia afzelii*, an endemic
76 cause of Lyme disease in Europe [29], and the microbiome of *Ixodes ricinus*, its primary vector.
77 We found that disrupting the microbiome of larval *I. ricinus* ticks by bleaching egg casings has
78 profound yet transient effects on the tick microbiota, but no effect on subsequent colonization by
79 *B. afzelii* when ticks feed on *B. afzelii*-infected hosts. In contrast, feeding on *B. afzelii*-infected
80 mice has pervasive effects on the composition and diversity of the tick microbiome, which are
81 largely independent of prior microbiome disruption. This suggests that *B. afzelii* infection
82 dynamics within rodent hosts have strong potential to sculpt the tick microbiota, with unclear
83 consequences for the transmission of other tick-borne pathogens. Negative interactions between
84 *B. afzelii* and other members of the tick microbiome might provide insights into the biological
85 control of tick-borne diseases.

86

87 **Methods**

88 ***Borrelia*, ticks, and mice:** We used *Borrelia afzellii* isolate NE4049, which was obtained
89 from an *I. ricinus* nymph at a field site near Neuchâtel, Switzerland. This strain has multi-locus

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90 sequence type ST679, *ospC* major group allele A10, and strain ID number 1887 in the *Borrelia*
91 MLST database. We used isolate NE4049 because we have previously shown that it is highly
92 infectious for both mice and ticks [30-32]. Pathogen-free, female *Mus musculus* BALB/c ByJ
93 mice were used as the vertebrate reservoir host. To infect mice via tick bite, we used *Ixodes*
94 *ricinus* nymphs infected with isolate NE4049 that had been generated in a previous study [30]
95 and that came from our laboratory colony of *Borrelia*-free *I. ricinus* ticks. For these *I. ricinus*
96 nymphs, the percentage of nymphs infected with *B. afzelii* ranged between 80.0% and 100.0%.
97 Uninfected control nymphs were obtained from our laboratory colony of *Borrelia*-free *I. ricinus*
98 ticks.

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

107 The remaining larvae for each of the 20 batches were split into two groups of ~100
108 larvae. For each of the 20 batches of eggs (10 tick families x 2 egg washing treatments), one
109 group of larvae was fed on an uninfected control mouse (n = 20 control mice) whereas the other
110 group of larvae was fed on a *B. afzelii*-infected mouse (n = 20 infected mice; **Figure 1**). These
111 mice had been infected with *B. afzelii* (or not) via nymphal tick bite (see below for details). The
112 resultant engorged larvae were placed in individual Eppendorf tubes and allowed to moult into

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113 nymphs. Four weeks after the larva-to-nymph moult, 10 nymphs were randomly selected from
114 each mouse and were frozen at –80°C (**Figure 1**). In summary, we froze 400 nymphs (10
115 families*2 egg washing treatments* 2 mice infection statuses*10 nymphs/mouse).

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

127 **Molecular methods for larval ticks:** The 20 groups of larval ticks that had been frozen
128 were split into two sub-groups with ~200 larval ticks per sub-group. Half of these sub-groups
129 were washed with ethanol prior to DNA extraction and the other sub-groups were not washed.
130 DNA extraction of the 40 sub-groups of larval ticks was done using a QIAGEN kit following the
131 manufacturer's instructions. The DNA of each sub-group was eluted into 100 µl of distilled
132 water. The DNA concentration was measured for each of the 40 sub-groups using a Nanodrop.
133 For qPCR, the DNA concentration of each sub-group was adjusted to 5 ng/µl. Two qPCR assays
134 were performed independently for each DNA extraction: tick *calreticulin* and bacterial *16S*
135 *rRNA*. Each qPCR assay contained 3 µl of template for a total of 15 ng of DNA.

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

145 **Illumina Library Preparation and Sequencing of the *16S rRNA* gene:** Of the 400
146 nymphs for which we had quantified bacterial load using the *16S rRNA* gene qPCR assay,
147 Illumina sequencing was performed for 360 nymphs. Sample preparation consisted of two PCR
148 reactions. In the first reaction we amplified a 464 bp fragment of the V3-V4 region of the *16s*
149 *rRNA* gene using primers Bakt_341F (5´CCTACGGGNGGCWGCAG-3') and Bakt_805R
150 (5´GACTACNVGGGTATCTAATCC-3') [33], designed with Illumina adapters. Reactions were
151 performed in a final volume of 50 µl using 2.5 U of HotStar HiFidelity DNA polymerase
152 (Qiagen,Germany), 2.5 µl of 10 µM primers, 10 µl of 15 µM dNTP mix, with a thermal cycle
153 with a denaturation step of 95°C for 5 min, 45 cycles of 94°C for 15 sec, 51°C for 45 seconds,
154 and 72°C for 45 seconds, with a final elongation step at 72°C for 7 minutes. Amplicons were
155 purified with the Wizard SV Gel and PCR Clean-Up system (Promega Switzerland).

156 The second PCR incorporated the sample barcodes. Reactions were performed in a final
157 volume of 25 µl using 1.25 U of HotStar HiFidelity DNA polymerase, 1 µl of 10 µM primers, 5
158 µl of 15 µM dNTP mix. The thermocycler had a denaturation step of 95°C for 5 min, 12 cycles

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159 of 95°C for 30 sec, 55°C for 30 seconds, and 72°C for 30 seconds, with a final elongation step of
160 72°C for 5 minutes, and amplicons purified as above. The 360 purified amplicons were pooled in
161 equimolar concentration using a Qubit 2.0 fluorometer (Invitrogen) and sequenced by
162 Microsynth (Balgach, Switzerland) using an Illumina MiSeq v2 with 250 bp paired end output,
163 followed by adaptor and quality trimming.
164

165 **Statistical Methods**

166 **Analysis of the bacterial load in the larvae:** For each sub-group of larval ticks, we
167 divided the *16S rRNA* gene copy number by the *calreticulin* gene copy number. These *16S rRNA*
168 to *calreticulin* ratios were log10-transformed to improve the normality of the data. The log10-
169 transformed *16S rRNA* to *calreticulin* ratios were analyzed using linear mixed effects models
170 (LMMs). Fixed factors included egg washing treatment (2 levels: water and bleach), larval tick
171 washing treatment (2 levels: none and ethanol), and their interactions. Random factors included
172 tick family. We used R/Bioconductor (v 3.4.2. or above) for analyses, including the *lme4*,
173 *complexHeatmap*, *vegan,* and *phyloseq* packages [34-37].

174 **Data selection of the nymphs:** Of the 370 DNA extractions, 14 were not included in the
175 analysis because their DNA concentrations were too low. After adjustment of the DNA
176 concentration, the remaining 356 DNA extractions had a DNA concentration that ranged
177 between 3.33 and 5.00 ng/µl so that the 3 µl of DNA template contained between 10.0 and 15.0
178 ng of DNA.

179 **Analysis of the bacterial load in the nymphs:** For each tick, we divided the *16S rRNA*
180 gene copy number by the *calreticulin* gene copy number. These ratios were log10-transformed to
181 normalize the data. The log10-transformed *16S rRNA* to *calreticulin* ratios were analysed using

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182 LMMs. Fixed factors included egg washing treatment (2 levels: water and bleach), *B. afzelii*
183 infection status of the mouse (2 levels: uninfected control, infected), nymphal tick washing
184 treatment (2 levels: none and ethanol), and their interactions. Random factors included tick
185 family and mouse identity nested inside tick family.

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

194 **Analysis of the *B. afzelii* spirochete load in the nymphs:** This analysis was restricted to
195 the subset of nymphs that were infected with *B. afzelii*. For each tick, we divided the *B. afzelii*
196 *flagellin* gene copy number by the tick *calreticulin* gene copy number. These ratios were log10-
197 transformed to normalize the data. The log10-transformed *flagellin* to *calreticulin* ratios were
198 analysed using LMMs. Fixed factors included egg washing treatment, nymphal tick washing
199 treatment, and their interaction. Random factors included tick family and mouse identity nested
200 inside tick family.

201 **Analysis of 16S rRNA amplicons:** Because of limited overlap between the 250 bp read
202 ends, we elected to use an OTU picking strategy that did not require first assembling paired end
203 reads into contigs. We generated OTU tables using the CD-HIT-OTU-Miseq workflow [38]
204 packaged with CD-HIT v 4.6.8. Forward and reverse read lengths were specified at 200 and 150

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205 bp and clustered against the SILVA 132 99% OTU release [39], otherwise using default
206 parameters that included using Trimmomatic for read trimming [40]. This identified 10,454
207 OTUs, although the vast majority of reads (93.6%) recruited to the 100 most abundant OTUs. It
208 also assigned taxonomy to only 926 OTUs (< 10%): for more robust taxonomic assignments, we
209 applied Metaxa2 v2.2 [41] to the representative sequences for each OTU identified by CD-HIT
210 using default parameters and the included reference database in Metaxa2. This identified 7,550
211 OTUs as bacterial and provided taxonomies that were largely congruent with CD-HIT
212 assignments, where evaluable; subsequent analyses were restricted to this bacterial OTU set.
213 The OTU table and taxonomy were imported to R (>v. 3.4.2), and analyzed using the
214 *phyloseq*, *vegan,* and *DeSeq2* packages (as detailed in Results). Linear mixed models for
215 univariable outcomes were implemented using the *lme4* package with a family as a random
216 effect, or nesting mouse host within tick family. In analyses using *DeSeq2* and *db-RDA*, family
217 was included as a fixed factor (*DeSeq2*) or a conditioning variable (*db-RDA*). We evaluated the
218 reliability of our replicated sequencing/analysis approach on the same nymphal samples via the
219 variance explained by sample in *db-RDA*, and the intraclass correlation coefficient for Shannon
220 diversity. For statistical analyses requiring tick infection status, we imputed missing infection
221 status for one *Borrelia*-exposed nymph as infected, as that was the most common state of
222 exposed nymphs.
223
224 **Results**
225 *Egg bleaching profoundly disrupts the microbiome of tick larvae*
226 Egg bleaching had strong effects on the abundance of bacteria associated with tick larvae,
227 as shown by a 27.5-fold reduction in the relative *16S rRNA* gene (hereafter *16S*) copy number

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228 (scored via qPCR) in larvae hatching from bleached versus unbleached eggs (N = 29 evaluable
229 samples; linear mixed model (LMM) controlling for tick family; $P < 10^{-6}$; $\log_{10}$ ratio of
230 *16S*/*calreticulin* 0.24 versus 6.61, respectively; **Figure 2A**). This reduction was evident six
231 weeks after treatment, demonstrating a profound effect of bleaching on the larval tick
232 microbiome. In contrast, washing larvae with ethanol prior to DNA extraction, which is expected
233 to reduce the external microbiota [but see 42], did not have a significant effect on the *16S* copy
234 number (LMM; P = 0.12; **Figure 2A**). Taken together, these observations suggest that the egg
235 bleaching-induced reduction of *16S* copy number was driven by an increase in the relative
236 abundance of the internal microbiota of the larval ticks at the expense of the external microbiota.
237 Multivariate analysis of the larval tick microbiome using db-RDA revealed that egg
238 bleaching led to a clear shift in the *16S* community, whereas there was no discernible effect of
239 washing the larvae prior to DNA extraction (db-RDA; based on Bray-Curtis dissimilarities of
240 $\log_{10}$ OTU abundances, stratified by tick family. Permutation tests; P < 0.001 and 0.727,
241 respectively; **Figure 2B**). Consistent with our expectations, the most significantly
242 (proportionally) enriched taxon due to bleaching was in the order Rickettsiales, which we further
243 manually annotated to *Candidatus* Midichloria mitochondrii, an endosymbiont of *I. ricinus* that
244 we expect would be unaffected by external bleaching (**Figure 2C**; $P_{adj} < 10^{-6}$; shown are the $\log_{10}$
245 read counts per thousand mapped reads). In contrast, egg bleaching significantly reduced the
246 relative abundance of Pseudomonas OTU 23 in the resultant larvae, which suggests that this
247 bacterium is found on the surface of the eggshell (**Figure 2C**; $P_{adj} < 10^{-3}$. Other significant
248 effects associated with bleaching included an increase in *Methylobacterium,* a taxon that has
249 been implicated as a potential contaminant of laboratory reagents [43]; these are consistent with
250 the strong reduction in *16S* abundance we observed via qPCR. No OTUs changed significantly

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251 from washing (all $P_{adj} > 0.05$). In sum, egg bleaching dramatically decreased *16S* copy number
252 and shifted the microbial community composition in larvae measured at 6 weeks after treatment.
253 Egg bleaching probably reduced the relative abundance of bacteria associated with the egg
254 surface and thereby increased the relative abundance of endosymbiont bacteria. The lack of an
255 observed effect of washing the larvae—on both microbial abundance and microbial diversity—
256 suggests that external microbiota are a minor component of the *16S* diversity in the lab-hatched
257 tick larvae.
258
259 *Manipulation of the larval microbiome has no effect on the acquisition of B. afzelii, and the*
260 *microbiome largely recovers in the unfed nymphs*
261 We screened nymphs for *B. afzelii* infection via qPCR and found no evidence that
262 disruption of the larval microbiome affected either the percentage of ticks that acquired the
263 infection while feeding (with infection prevalences of 68.2% and 73.5% in unbleached and
264 bleached groups respectively; binomial GLMM; P = 0.44, N = 183, **Figure 3A**) or *B. afzelii* copy
265 number in the nymphs that became infected (LMM; P = 0.958, N = 130; **Figure 3B**). Thus,
266 although the egg bleaching treatment was highly effective at reducing the microbiome in larvae,
267 it did not affect the ability of *B. afzelii* to colonize ticks during or following the larval blood
268 meal.
269 Although the egg bleaching treatment strongly reduced the *16S* copy number in the
270 larvae, the bacterial community largely recovered after these larvae had taken a blood meal and
271 molted into nymphs, as there was no effect of the egg bleaching on *16S* copy number in nymphs
272 (LMM; P = 0.272, N = 356, **Figure 3C**). Intriguingly, in contrast to the non-significant effect of
273 ethanol washing on *16S* copy number that we observed in larvae, there was a clear reduction

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274 with both washing and *B. afzelii* exposure. The *16S* copy number was lowest in ticks that were
275 both washed and *B. afzelii*-exposed (LRT for interaction; $P < 10^{-5}$), indicating that feeding on *B.*
276 *afzelii*-infected mice reduced the internal bacterial load in nymphs; we investigate this hypothesis
277 in more detail below.
278

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

280 To extend our analysis of larval ticks to the nymphal stage, we used *16S* amplicon
281 sequencing to profile replicate nymphs from each treatment alongside the larval samples (above).
282 We again restricted these analyses to the 40 most abundant OTUs. As expected from prior
283 reports [44, 45], there was a clear shift in the tick-associated bacterial community from larval to
284 nymphal ticks (**Figure 4A**). There was, further, a striking effect of *B. afzelii* infection in the
285 mouse on the tick microbiota as quantified in the nymphal stage, with pronounced differences
286 between ticks fed on *B. afzelii*-infected versus control mice (db-RDA; $P < 0.001$; **Figure 4B**
287 shows unsupervised principal coordinates analysis (PCoA)). We also found that egg bleaching
288 and ethanol washing prior to DNA extraction had modest but significant effects on microbiome
289 composition in nymphs ($P < 0.001$ and $P = 0.014$, respectively; **Figure 4B**), consistent with our
290 finding that washing the nymphs decreased *16S* copy number. While sequencing recovered
291 OTUs annotated as *Borrelia*, the most abundant *Borrelia*-annotated OTU was ranked 81st in
292 overall abundance and accounted for <0.05% of total sequence reads. Thus, a direct contribution
293 of sequenced *Borrelia 16S* amplicons in infected ticks does not explain these strong patterns.

294 Microbiome α-diversity (measured as Shannon entropy) in nymphs modestly increased as
295 a result of both microbiome disruption via egg bleaching (LMM; $P = 0.035$; $N = 305$. **Figure**
296 **5A**) and from *B. afzelii* infection ($P = 0.0025$; **Figure 5B**) or exposure ($P = 0.0007$; competing

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297 multivariable model; **Figure 5B**). Coupled with the decrease in *16S* copy number observed when
298 feeding on *B. afzellii*-infected mice, these diversity effects appeared to be mediated through
299 disproportionately negative impacts on abundant OTUs (e.g., *Stenotrophomonas*) leading to
300 increased community evenness. As mentioned, these trends were evident when considering the
301 infection status of the tick itself, or that of the mouse on which they were fed (**Figure 5C**).
302 Comparison between control nymphs, uninfected nymphs, and infected nymphs demonstrated
303 that it was feeding on an infected mouse rather than acquiring *B. afzelii* infection that was most
304 important for determining the nymphal microbiome (**Figure 5C**). This was supported by
305 comparing competing models with Akaike's Information Criterion (AIC), which found that
306 feeding on an infected mouse was a much stronger predictor of both α-diversity (ΔAIC ~ 6) and
307 the multivariate bacterial community (**Figure 5A;** ΔAIC ~14 in models using first PCoA axis as
308 response variable) than acquisition of *B. afzelii* by the tick (**Figure 5**), suggesting that the effects
309 we observe are more likely to be caused by physiological or immunological characteristics of
310 infected mice rather than the direct effects of *B. afzelii* infection in the ticks. Similarly,
311 examining the OTU frequencies in bleached and *B. afzelii*-exposed ticks showed that these net
312 effects were driven by proportional reductions in the dominant OTUs, with concomitant
313 increases in less frequent OTUs (e.g. **Figure 4**). Collectively, these results are consistent with the
314 strong effects of mouse *B. afzelii* infection status we observe on *16S* copy number and suggest
315 that these are specifically mediated by disproportionate negative effects on abundant bacterial
316 OTUs.
317
318 *Microbial correlates of Borrelia exposure*

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319 To characterize the recurrent shifts in the tick microbiome associated with *B. afzelii*
320 exposure, we used negative binomial models implemented in *phyloseq/DESeq2*. In line with the
321 global diversity shifts we observed, feeding on *B. afzelii*-infected mice led to significant changes
322 in the relative frequencies of many OTUs (with 19/40 significant at $P_{adj} < 0.05$; Figure 5). This
323 was most evident in large decreases in the frequency of multiple *Stenotrophomonas* OTUs and
324 other Gammaproteobacteria (**Figure 6**). As a result, there appeared to be a degree of taxonomic
325 dependence in microbial responses to *B. afzelii* exposure, with Betaproteobacteria generally
326 significantly increasing in frequency and Gammaproteobacteria decreasing (Fisher's exact test, P
327 $= 0.004$; **Figure 6**). Our analysis also revealed that several nymph OTUs that increased in
328 frequency in response to tick bleaching, including two *Burkholderia* OTUs (Betaproteobacteria;
329 $P_{adj} < 0.01$; **Figure 6**) and a *Bradyrhizobium* OTU (Alphaproteobacteria; $P_{adj} < 0.01$; **Figure 6**),
330 whereas no OTUs significantly (proportionally) decreased. Consistent with our expectations,
331 ethanol washing prior to DNA extraction increased the relative abundance of the endosymbiotic
332 *Candidatus* Midichloria mitochondrii (annotated as order *Rickettsiales*, Alphaproteobacteria) as
333 well as *Spiroplasma* (Mollicutes) by 2.5 and 1.7-fold respectively ($P_{adj} < 0.01$), corroborating an
334 enriching effect of washing on the internal tick microbiota.
335
336 **Discussion**
337 Here, we used a highly replicated experiment on wild-collected tick families to examine
338 the reciprocal interactions between the endogenous tick microbiota and *B.afzelii.* We used egg
339 bleaching to radically disrupt the microbiome in larval ticks but found no evidence that this
340 affected the subsequent susceptibility of ticks to infection with *B. afzelii*. Rather, this work
341 revealed striking effects of feeding on *B. afzelii*-infected mice on the tick microbiome; these

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342 effects superseded those of actually acquiring an infection, as judged by comparing competing
343 statistical models, and the resultant partitioning of samples in microbial community space (e.g.,
344 **Figure 4A, 5C**).

345 A growing number of studies have investigated whether dysbiosing ticks influences their
346 susceptibility to acquiring tick-borne pathogens [8, 11-13]. In some systems, microbiome
347 disruption makes tick species more susceptible to infection with tick-borne pathogens [8, 13],
348 whereas other systems found the opposite effect [11, 12]. In the present study, we found no
349 evidence that microbiome disruption influenced *B. afzelii* infection rates or the *B. afzelii*
350 spirochete load in infected ticks. In contrast, a previous study found that dysbiosed *I. scapularis*
351 larvae were less likely to acquire *B. burgdorferi* ss and contained lower bacterial loads compared
352 to control larvae [12]. These two studies differed in a number of factors including the *Borrelia*
353 species, the *Ixodes* tick species, and the method of dysbiosis. An important aspect of the present
354 study is that we investigated whether dysbiosis of the eggs influenced the infection status of the
355 nymphs, which is the stage that is actually critical for the transmission of Lyme disease in nature
356 [46].

357 Despite the fact that bleaching the eggs was highly effective at reducing the bacterial
358 microbiome in the resultant larvae, this method of 'dysbiosis' did not have a meaningful impact
359 on *B. afzelii* transmission. While the presence of additional bacteria to those uncovered here
360 could influence these patterns, we found little influence of tick family on the recovered bacterial
361 communities, outside of potentially vertically transmitted bacteria such as *Spiroplasma*,
362 suggesting the microbial community of *I. ricinus* is largely homogenous at the scale studied here.
363 Our method of dysbiosis further highlights the neglected importance of maternal transmission of
364 gut symbionts in ticks. In arthropods, gut symbionts are typically vertically transmitted by

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365 superficial bacterial contamination of eggs (egg smearing) [47]. Our egg washing with bleach
366 has removed such maternally inherited gut symbionts and this impacted the microbial
367 communities hosted by larvae. Our study shows that egg smearing is a key mechanism for
368 colonization of ticks by their associated microbes.

369 *B. afzelii* infection reduced the microbial abundance (in the ethanol-washed nymphs) and
370 changed the microbial community in the unfed nymphs. Notably, we observed changes in OTU
371 relative abundance to be, at least in part, taxon-specific with decreases in Gammaproteobacteria
372 and increases in Betaproteobacteria (**Figure 6**). The observation that mouse infection status was
373 more important than tick infection status suggests that the blood physiology at the time of the
374 larval blood meal was critical for structuring the subsequent nymph microbiome. Metabolomic
375 studies of mouse serum samples have shown that *B. burgdorferi* ss infection changes the blood
376 concentration of amino acids, energy metabolites, and aromatic compounds [48], which could
377 influence the development of the tick microbiome. Infection with *B. burgdorferi* sl stimulates the
378 host immune system, which could also exert collateral damage on the tick microbiome [49-52].
379 For example, elevated levels of complement, cytokines, leukocytes, and reactive oxygen species
380 in the blood [52-55] may interact inside the tick to have negative effects on the midgut
381 microbiome. In summary, our study suggests that the physiological and immunological changes
382 associated with infection in the vertebrate host have important consequences for the microbiome
383 of feeding ticks.

384

385 **Conclusions**

386 In summary, we found that egg bleaching resulted in a 30-fold reduction of the microbiome of
387 larval ticks. This microbiome manipulation had no effect on the ability of larval ticks to acquire

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388 *B. afzelii* after feeding on infected mice. Once the engorged larvae had moulted into unfed
389 nymphs, the dramatic effect of the egg bleach treatment on the tick microbiome had mostly
390 disappeared. The *B. afzelii* infection status of the mice that provided the larval blood meal had a
391 dramatic effect on the microbiome of the resultant unfed nymphs. Our study suggests that
392 infection in the vertebrate host influences the quality of the larval blood meal with long-term
393 consequences for the tick microbiome that persist into the nymphal stage.
394
395
396 **Ethics approval and consent to participate:** The commission that is part of the "Service de la
397 Consommation et des Affaires Vétérinaires (SCAV)" of Canton Vaud, Switzerland evaluated
398 and approved the ethics of this study. The Veterinary Service of the Canton of Neuchâtel,
399 Switzerland issued the animal experimentation permit used in this study (NE04/2014).
400
401 **Availability of data and materials:** Raw sequencing reads will be deposited at NCBI (accession
402 pending). Supplementary data and scripts to reproduce the analysis will be made available at
403 github.com/onecarbon/tickdysbiosis (pending).
404
405 **Competing interests:** The authors declare that they have no competing interests.
406
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411

412 **Authors' contributions:** EM, OD, OP and MJV conceived and designed the study. EM, AS, and
413 AB conducted the experiment and performed the molecular work. PTH and MJV analysed the
414 data and wrote the manuscript. GH created the figure of the experimental design. All authors
415 read and approved the final version of the manuscript.

416

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586
587 **Figure Legends:**
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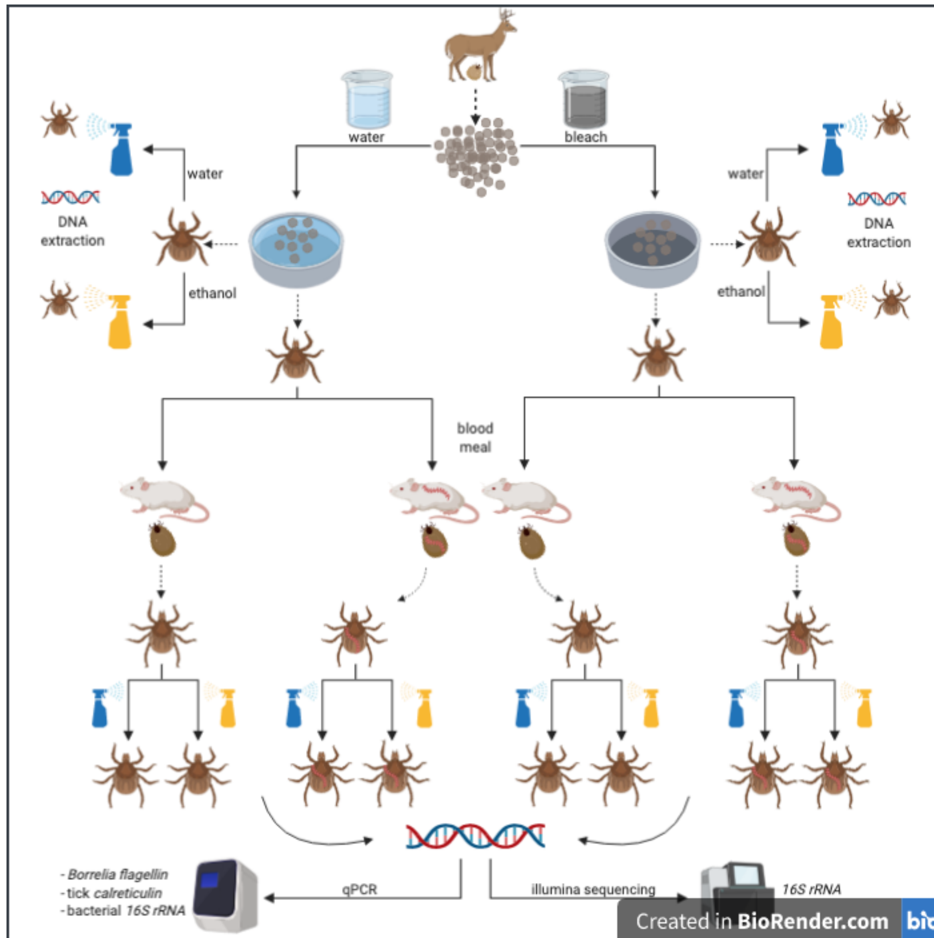

590

591
592 **Figure 1.** Experimental design. Engorged female *I. ricinus* ticks (n = 10) were collected from roe
593 deer captured in the Chizé forest, France and laid their eggs in the laboratory. Each of the 10 egg
594 batches was split into two batches and rinsed with either 10% bleach (n = 10 batches) or distilled
595 water (n = 10 batches) and hatched into larvae. To determine whether the egg bleaching
596 treatment reduced the microbiome, a subset of larvae was tested using qPCR and Illumina
597 sequencing of the bacterial *16S rRNA* gene. Larvae for each of the 20 batches were split into two
598 groups of ~100 larvae. For each of the 20 batches of eggs (10 tick families x 2 egg washing
599 treatments), one group of larvae was fed on an uninfected control mouse (n = 20 control mice)
600 whereas the other group of larvae was fed on a *B. afzelii*-infected mouse (n = 20 infected mice).
601 Engorged larvae were placed in individual Eppendorf tubes to moult into nymphs. Four weeks
602 after the moult, 10 nymphs were randomly selected from each of the 40 mice and frozen at –
603 80°C (n = 400 nymphs). These nymphs were tested for *B. afzelii* infection using qPCR and for
604 their bacterial load and microbiome using qPCR and Illumina sequencing of the bacterial *16S*
605 *rRNA* gene.
606

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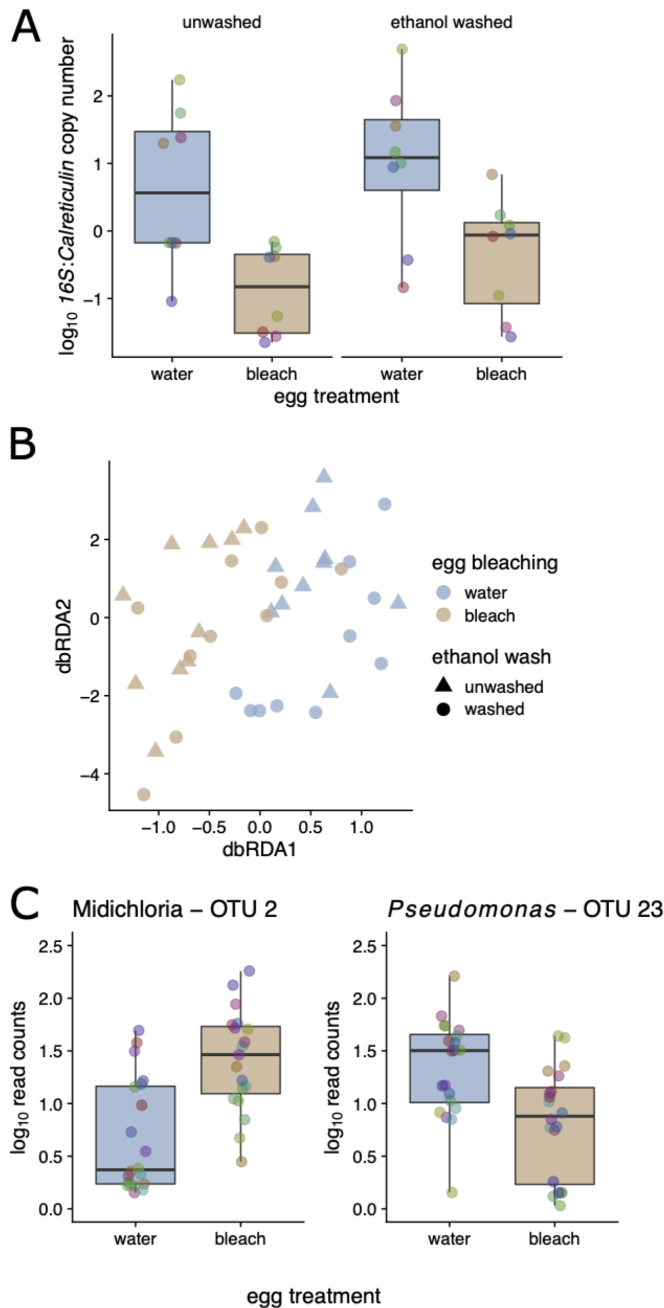

608
609 **Figure 2. Bleaching of egg casings profoundly decreases the abundance of microbiota in**
610 **larval ticks.** Pooled *Ixodes scapularis* larvae were quantified six weeks after egg bleaching. **A.**
611 Egg bleaching led to a ~27.5-fold reduction in the relative copy number of the 16S rRNA in
612 larvae (vs tick *calreticulin*; $P < 0.001$), irrespective of ethanol washing prior to DNA extraction
613 ($P > 0.05$). **B.** Egg bleaching, but not washing with ethanol prior to DNA extraction, led to
614 significant shifts in the bacterial community, as measured by 16S amplicon sequencing and
615 dbRDA ($P < 0.001$ and $P > 0.05$, respectively). **C.** The most enriched taxon in response to egg
616 bleaching was *Candidatus* Midichloria mitochondrii (Padj < 0.001); in contrast the most depleted
617 was a *Pseudomonas* OUT (the Y-axis has units of number of read counts per thousand mapped

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618 reads). Colored points in boxplots represent individual data points (pooled larval samples,
619 colored by the tick mother; 'family'; N = 10).
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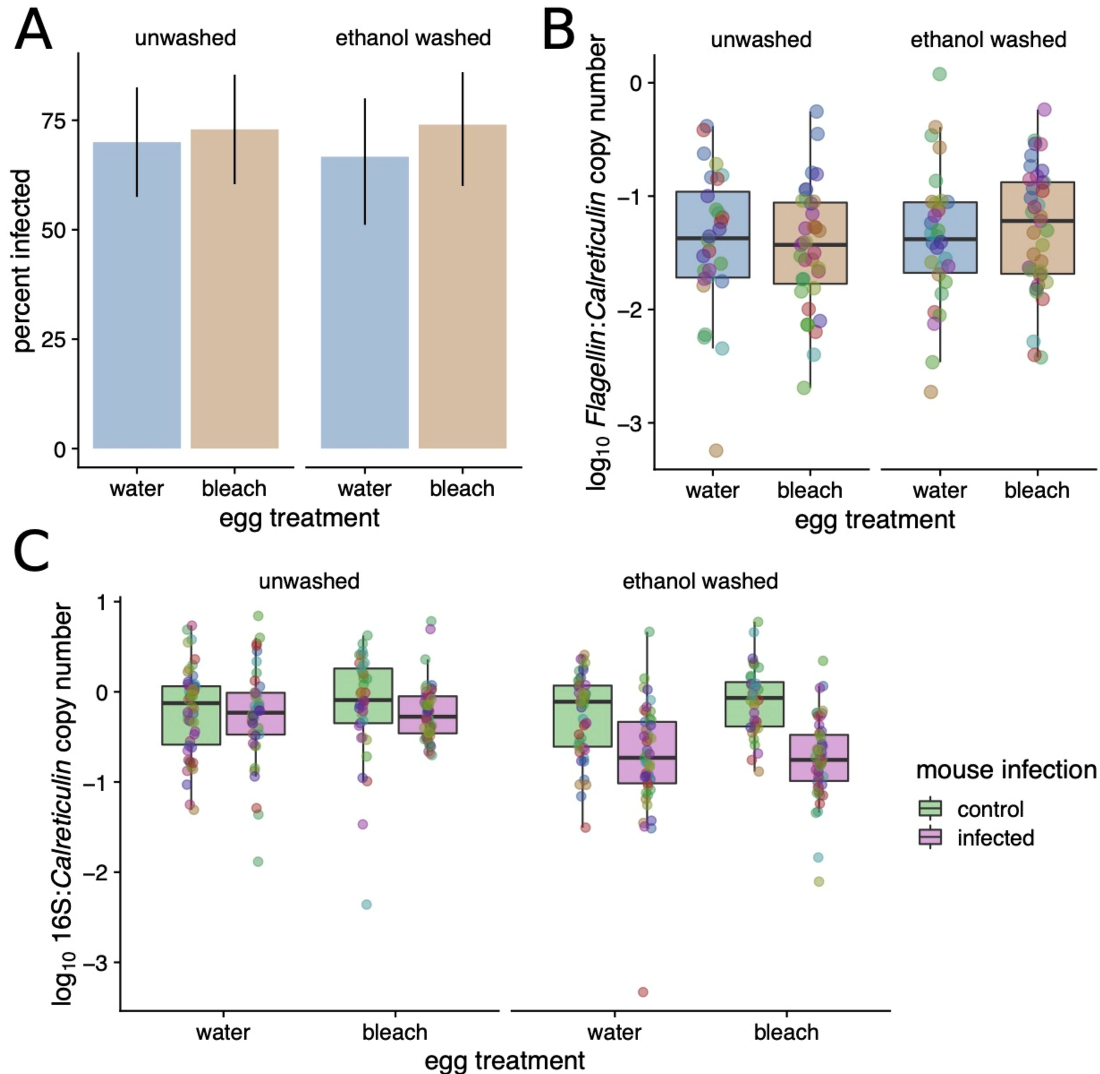

623

624 **Figure 3. 'Dysbiosing' larval ticks does not affect infection success by *B. afzelii*.** Neither **A.**
625 the percentage of nymphs that acquired *B. afzelii* during their larval blood meal nor **B.** the
626 inferred *B. afzelii* load in these nymphs were affected by prior microbiome disruption (all P >>
627 0.05). **C.** Feeding on *B. afzelii*-infected mice decreased the bacterial load in *I. ricinus* nymphs;
628 this effect was more visible in the nymphs that were washed prior to DNA extraction compared
629 to the unwashed nymphs.

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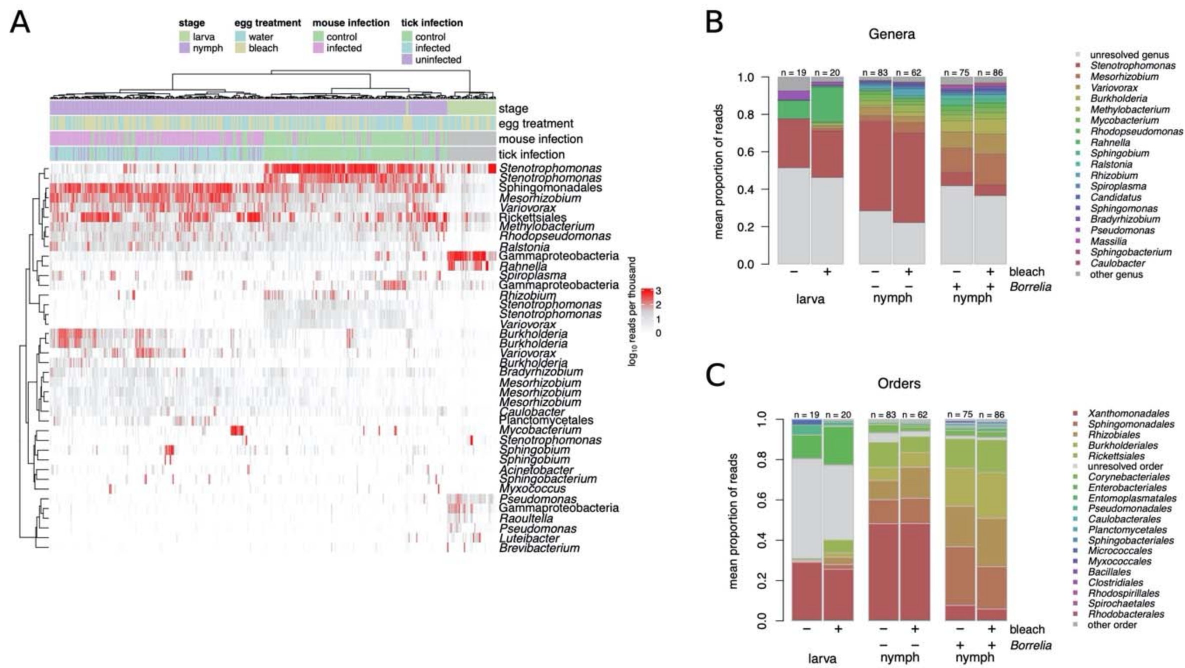

630

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

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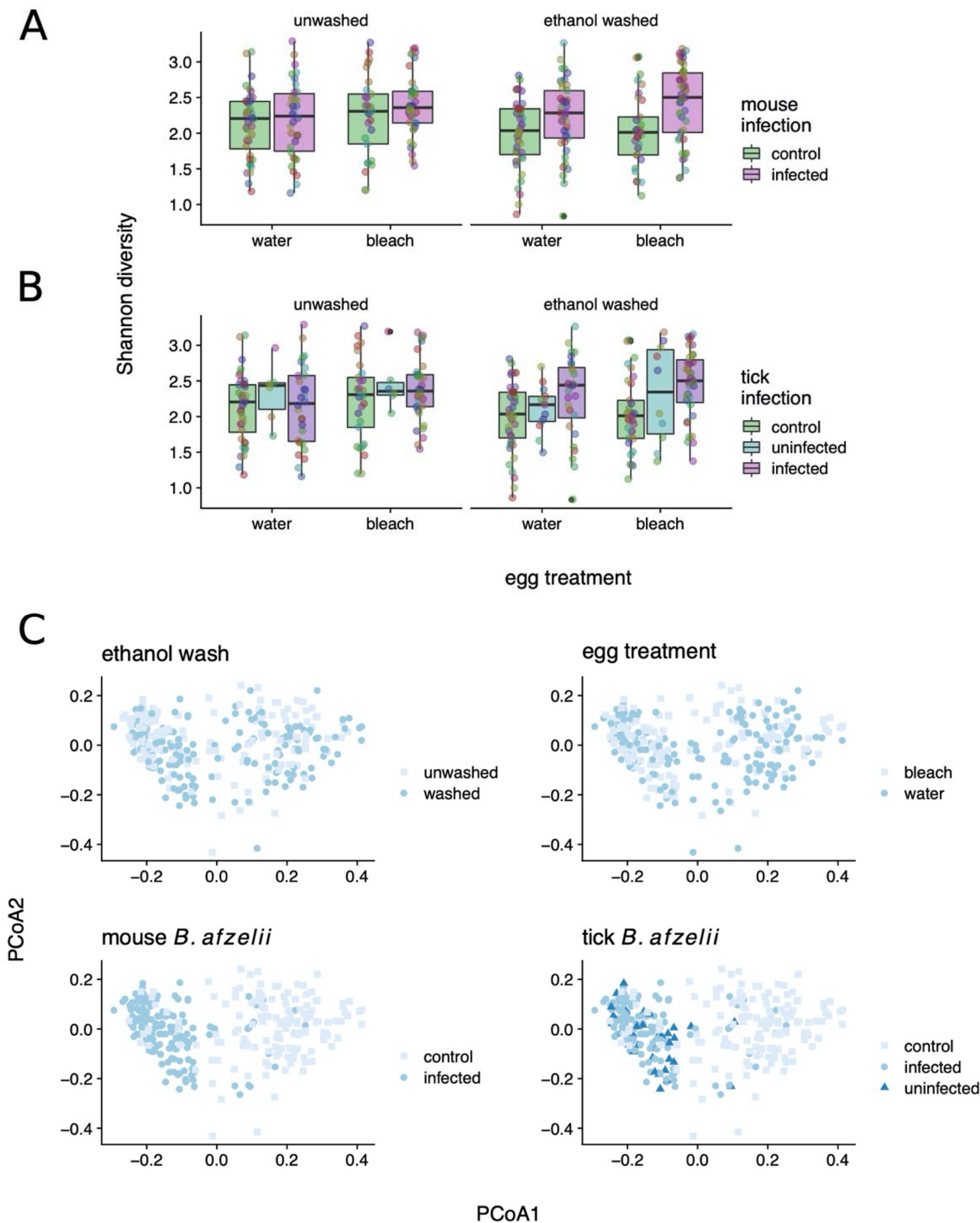

637

638 **Figure 5. Egg bleaching and rinsing and *B. afzelii* exposure increase the diversity of the 16S**
639 **microbiome of *I ricinus* nymphs.** Shannon diversity increases in both **A.** *B. afzelii* exposed
640 ticks and **B.** *B. afzellii* infected ticks (P <0.01). Akaike's Information Criterion (AIC) shows
641 exposure to be a stronger predictor of Shannon diversity than infection (deltaAIC ~6) in linear
642 mixed models. Points represent individual tick nymphs colored by family of origin. C. Principal
643 Coordinates Analysis of nymphal 16S counts, colored by experimental factors; *B afzelii* exposure
644 best stratifies groups on PCoA 1 (deltaAIC ~11 vs infection).
645

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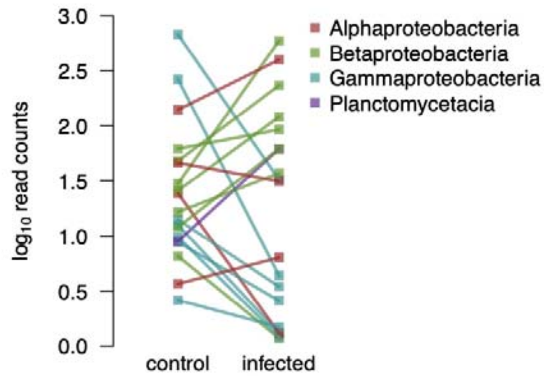

646
647
648 **Figure 6.** Proportional abundance of taxa that changed significantly in *I. ricinus* nymphs (19/40
649 at $P_{adj} < 0.05$) in response to feeding on *B. afzelii* infected mice, shown at the level of class.
650 There is significant taxonomic dependence (Fisher's exact test $P = 0.004$) of taxonomic response.
651
652

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653 **Supplemental Material:**
654
655 **Data S0:** Description of molecular methods
656
657 **Data S1:** Metadata
658
659 **Data S2:** Metadata
660
661 **Data S3:** OTU table produced by CD-HIT-OTU_MiSEQ.
662
663 **Data S4:** OTU clusters produced by CD-HIT-OTU_MiSEQ.
664
665 **Data S5:** OTI taxonomy assigned by Metaxa2.
666
667