

1 3 figures, 2 tables, 7345 words

2 **Tardigrade community microbiomes in North American orchards include putative**  
3 **endosymbionts and plant pathogens**

4 **Laura E. Tibbs-Cortes**\*<sup>1,2</sup>, **Bienvenido W. Tibbs-Cortes**<sup>3,4</sup>, **Stephan Schmitz-Esser**<sup>3,4</sup>

5 <sup>1</sup>Department of Agronomy, Iowa State University, Ames, IA, USA

6 <sup>2</sup>Interdepartmental Genetics and Genomics Program, Iowa State University, Ames, IA, USA

7 <sup>3</sup>Department of Animal Science, Iowa State University, Ames, IA, USA

8 <sup>4</sup>Interdepartmental Microbiology Program, Iowa State University, Ames, IA, USA

9 **\*Correspondence:**

10 Laura E. Tibbs-Cortes

11 [ltibbs@iastate.edu](mailto:ltibbs@iastate.edu)

12 **Keywords: tardigrade, microbiota, phytopathogen, endosymbiont, amplicon sequencing**

13 **Abstract**

14 The microbiome of tardigrades, a phylum of microscopic animals best known for their  
15 ability to survive extreme conditions, is poorly studied worldwide and completely unknown in  
16 North America. An improved understanding of tardigrade-associated bacteria is particularly  
17 important because tardigrades have been shown to act as vectors of the plant pathogen  
18 *Xanthomonas campestris* in the laboratory. However, the potential role of tardigrades as  
19 reservoirs and vectors of phytopathogens has not been investigated further. This study analyzed  
20 the microbiota of tardigrades from six apple orchards in central Iowa, USA, and is the first  
21 analysis of the microbiota of North American tardigrades. It is also the first ever study of the  
22 tardigrade microbiome in an agricultural setting. We utilized 16S rRNA gene amplicon  
23 sequencing to characterize the tardigrade community microbiome across four contrasts: location,  
24 substrate type (moss or lichen), collection year, and tardigrades versus their substrate. Alpha  
25 diversity of the tardigrade community microbiome differed significantly by location and year of  
26 collection but not by substrate type. Our work also corroborated earlier findings, demonstrating  
27 that tardigrades harbor a distinct microbiota from their environment. We also identified  
28 tardigrade-associated taxa that belong to genera known to contain phytopathogens  
29 (*Pseudomonas*, *Ralstonia*, and the *Pantoea/Erwinia* complex). Finally, we observed members of  
30 the genera *Rickettsia* and *Wolbachia* in the tardigrade microbiome; because these are obligate  
31 intracellular genera, we consider these taxa to be putative endosymbionts of tardigrades. These  
32 results suggest the presence of putative endosymbionts and phytopathogens in the microbiota of  
33 wild tardigrades in North America.

34

## 35 1 Introduction

36 Tardigrades are a poorly-studied but globally ubiquitous phylum of microscopic animals.  
37 They are members of the superphylum Ecdysozoa, a group that also includes arthropods and  
38 nematodes. All tardigrades are aquatic; however, while some live in bodies of fresh or salt water,  
39 they are most commonly collected from moss or lichen, where they live in interstitial films of  
40 water. When this water dries up, tardigrades survive by dehydrating and entering a state of  
41 dramatically reduced metabolism known as cryptobiosis (Kinchin 1994). In this state, they are  
42 famously able to survive extreme conditions, ranging from temperatures near absolute zero  
43 (Becquerel 1950) to the vacuum of space (Jönsson et al. 2008). Despite extensive study of  
44 tardigrades' survival abilities, little is known about many aspects of their biology, including their  
45 microbiota (Vecchi et al. 2018). This is particularly important because tardigrades' presence in  
46 moss and lichen, which often grow on tree bark, brings them into close contact with trees,  
47 including important orchard crops such as apple trees (*Malus domestica* L. Borkh). Therefore,  
48 any plant pathogens present in the tardigrade microbiota have the potential to affect these crops,  
49 underscoring the importance of understanding the tardigrade microbiota in an agricultural  
50 context.

51 The first study of tardigrade-associated bacteria, published in 1999, found that bacteria of  
52 the phytopathogenic genus *Xanthomonas* could be grown from the feces of the tardigrade  
53 *Macrobiotus hufelandi* C.A.S. Schultze, 1834 isolated from the wild. However, attempts to  
54 inoculate *Mac. hufelandi* with *Serratia marcescens* were unsuccessful, suggesting a non-random  
55 relationship between *Mac. hufelandi* and *Xanthomonas* (Krantz, Benoit, and Beasley 1999). The  
56 following year, a second paper showed that *Mac. hufelandi* exposed to infected leaves could  
57 spread *X. campestris* pv. *raphani* (the causal pathogen of radish leaf spot disease) to healthy  
58 radish seedlings in the laboratory. This demonstrated that *Mac. hufelandi* can act as a vector of  
59 radish leaf spot disease (Benoit et al. 2000).

60 Animal vectors are known to spread many plant diseases, with major consequences for  
61 crop production worldwide (Ng and Falk 2006; Mew 1993; Duveiller, Bragard, and Marite  
62 1997). Current research focuses on insect vectors (Ng and Falk 2006), but the work of Krantz et  
63 al. and Benoit et al. demonstrate that at least one tardigrade species (*Mac. hufelandi*) can spread  
64 bacterial disease in plants (Benoit et al. 2000) and can act as reservoirs of plant pathogens  
65 (Krantz, Benoit, and Beasley 1999). Because *Mac. hufelandi* and many other tardigrade species  
66 live in close contact with plants, bacteria deposited in their feces may infect these plants,  
67 especially as other Ecdysozoans are known to spread phytopathogens in this manner (Dutta et al.  
68 2014; Stavrinides, McCloskey, and Ochman 2009). Tardigrades also have the potential to spread  
69 phytopathogenic bacteria over large areas because many tardigrade species are cosmopolitan  
70 (Meyer 2013) and may be dispersed by wind or migratory birds (Mogle et al. 2018).

71 The genus *Xanthomonas* found in association with *Mac. hufelandi* includes pathovars that  
72 infect staple food crops including rice (*Oryza sativa* L.) (Mew 1993), wheat (*Triticum aestivum*  
73 L.) (Duveiller, Bragard, and Marite 1997), and maize (*Zea mays* L.) (Karamura et al. 2007) with  
74 potentially devastating effects. For example, bacterial blight (*X. oryzae* pv. *oryzae*) can cause  
75 yield losses of up to 50% in rice infected as seedlings, impacting both economies and food  
76 security (Mew 1993). Yet, although tardigrades are known vectors of this important genus, there

77 has been no additional literature published on phytopathogens associated with tardigrades in the  
78 past two decades.

79         Recent studies of the tardigrade microbiome, while not focusing on phytopathogens, have  
80 leveraged advances in sequencing technology by using 16S rRNA gene amplicon sequencing.  
81 Vecchi *et al.* surveyed the microbial communities associated with six tardigrade species:  
82 *Acutuncus antarcticus* (Richters, 1904) collected from freshwater sediment in Antarctica, after  
83 which a subsample was raised in laboratory culture; *Ramazzottius oberhaeuseri* (Doyère, 1840),  
84 collected from lichen on two different trees in Italy; *Macrobotus macrocalix* Bertolani &  
85 Rebecchi, 1993 and *Richtersius coronifer* (Richters, 1903), both collected from the same moss  
86 on a rock in Sweden; and *Echiniscus trisetosus* Cuénot, 1932, and *Paramacrobotus areolatus*  
87 (Murray, 1907), both collected from the same moss on a rock in Italy. The authors found that the  
88 tardigrade microbiome is dominated by *Proteobacteria* and *Bacteroidetes*, is distinct from and  
89 usually less diverse than that of their substrates, differs among tardigrade species, and is altered  
90 by laboratory culturing of the tardigrades. Vecchi *et al.* also identified potential endosymbionts  
91 of the obligate intracellular order *Rickettsiales* within the tardigrade microbiome (2018). This is  
92 particularly intriguing because the genera *Wolbachia* and *Rickettsia*, both members of  
93 *Rickettsiales*, are known to have reproductive effects on their hosts, including inducing  
94 parthenogenesis (Giorgini *et al.* 2010; Werren, Baldo, and Clark 2008). Notably, parthenogenesis  
95 is common in tardigrades (Bertolani 2001; Guil *et al.* 2022). A subsequent analysis of these data  
96 identified four putative endosymbionts in the order *Rickettsiales*, three of which belonged to  
97 *Anaplasmataceae* and one to *Ca. Tenuibacteraceae*. These were differentially associated with  
98 different tardigrade species, and fluorescence *in situ* hybridization (FISH) detected bacteria  
99 within the ovaries of some tardigrades, suggesting that tardigrade endosymbionts are vertically  
100 transmitted (Roberto Guidetti *et al.* 2020).

101         A second study surveyed the microbiota of a newly-described tardigrade species,  
102 *Paramacrobotus experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020,  
103 collected from two samples of moss growing on soil in Madagascar and subsequently raised in  
104 laboratory culture for two years before DNA extraction (Kaczmarek *et al.* 2020). This study  
105 again identified differences between the tardigrades' microbiome and that of their environment  
106 and detected evidence of putative endosymbionts of the intracellular groups *Rickettsiales* and  
107 *Polynucleobacter*. *Proteobacteria* and *Firmicutes* were the dominant phyla in *Pam.*  
108 *experimentalis*, and 31 operational taxonomic units (OTUs) shared across tardigrade samples  
109 were identified as potential core microbiome members for this tardigrade species (Kaczmarek *et*  
110 *al.* 2020).

111         A third paper conducted 16S rRNA amplicon sequencing on four tardigrade species:  
112 *Hypsibius exemplaris* Gąsiorek, Stec, Morek & Michalczyk, 2018, collected from rotting leaves  
113 in a pond in the United Kingdom; *Macrobotus polypiformis* Roszkowska, Ostrowska, Stec,  
114 Janko & Kaczmarek, 2017, collected from moss on a wall in Ecuador; *Paramacrobotus*  
115 *fairbanksi* Schill, Förster, Dandekar & Wolf, 2010, collected from moss in Antarctica; and  
116 *Paramacrobotus* sp. Guidetti, Schill, Bertolani, Dandekar & Wolf, 2009, collected from moss  
117 on a wall, soil, and railroad tracks at two locations in Poland. Of these, all but *Pam. fairbanksi*  
118 were subsequently cultured prior to DNA extraction. This study identified *Proteobacteria*,  
119 *Firmicutes*, and *Actinobacteria* as the most abundant phyla in the studied tardigrades, but

120 primarily focused on putative endosymbionts of tardigrades, specifically OTUs assigned to  
121 *Rickettsiales* and *Wolbachia*. Members of *Wolbachia* were detected in adult *Pam.* sp. and *Mac.*  
122 *polypiformis*, and *Rickettsiales* were detected in eggs of *Pam. Fairbanksi* as well as adult *Mac.*  
123 *polypiformis* and *Pam.* sp. Neither *Rickettsiales* nor *Wolbachia* were detected in *Hys. exemplaris*  
124 or the adult *Pam. fairbanksi* (Mioduchowska et al. 2021).

125 Most recently, Zawierucha *et al.* sequenced 16S rRNA, ITS1, and 18S rRNA genes to  
126 identify bacteria, fungi, and microeukaryotes, respectively, associated with the glacial tardigrade  
127 *Cryobiotus klebelsbergi* (Mihelčič, 1959). *C. klebelsbergi* were collected from cryoconite on the  
128 surface of Forni Glacier in Italy; DNA was extracted from four samples immediately and from  
129 another three after starving for three weeks. The authors found that relative richness of bacteria,  
130 fungi, and microeukaryotes was highest in cryoconite, followed by fed tardigrades and finally  
131 starved tardigrades. *Polaromonas* sp. was the most abundant bacterium in both fed and starved  
132 *C. klebelsbergi*, while *Pseudomonas* sp. and *Ferruginibacter* sp. were the second most abundant  
133 bacteria in fed and starved tardigrades, respectively.

134 16S rRNA gene amplicon sequencing has allowed major advances in understanding of  
135 the tardigrade microbiota. However, contamination is an ongoing issue in microbiome studies,  
136 especially in low microbial biomass samples such as tardigrades where contaminants can make  
137 up a relatively large proportion of all sequence reads and therefore have a disproportionately  
138 large impact on results. A minimum standard developed for such studies is the RIDE checklist,  
139 which advises researchers to report the methodology used to reduce and assess contamination, to  
140 include three types of negative controls (sampling blank, DNA extraction blank, and no-template  
141 amplification controls), to determine contamination level by comparing these negative controls  
142 to the samples, and to explore contaminants' impact on results (Eisenhofer et al. 2019).  
143 However, while recommended laboratory practices can reduce contamination, they cannot  
144 eliminate it. Therefore, *in silico* approaches have been developed to better accomplish the last  
145 two steps of the RIDE checklist. For example, the program decontam identifies contaminants  
146 based on presence in negative controls and higher frequencies in low-concentration samples. It  
147 then removes them from further analysis, dramatically improving the accuracy of results (Davis  
148 et al. 2018; Karstens et al. 2019). In this work, we followed the RIDE checklist and utilized  
149 decontam for *in silico* contaminant removal.

150 This study represents the first survey of tardigrade microbiota in North America, as well  
151 as the first such survey in an agricultural setting (apple orchards). Rather than focusing on the  
152 microbiome of individual tardigrade species, this work is the first to study the microbiome of a  
153 full community of tardigrades, hereafter referred to as the tardigrade community microbiome. It  
154 is also only the fifth survey of the tardigrade microbiome ever conducted and leverages  
155 contamination mitigation methods not used in the previous studies. In addition to identifying  
156 putative plant pathogens and endosymbionts associated with tardigrade communities in apple  
157 orchards, this study examines whether the tardigrade microbiome differs in four contrasts: (1)  
158 across locations, (2) between substrates (moss vs. lichen), (3) between tardigrades and their  
159 substrates, and (4) across years.

## 160 **2 Materials and Methods**

### 161 **2.1 Moss and Lichen Sample Collection**

162 In summer 2019, lichen samples were collected from apple trees growing in six orchards  
163 (Locations 1-6) in Hardin and Franklin counties in north-central Iowa, USA (Fig. S1). One of  
164 these (Location 1) had previously been surveyed for tardigrades (Tibbs-Cortes, Tibbs-Cortes,  
165 and Miller 2020). One sample of lichen was collected from each tree, and three to five trees were  
166 sampled at each location. Moss was also present on the sampled apple trees at Location 2, so a  
167 moss sample was collected from three of these trees. In June 2020, additional lichen samples  
168 were taken from the trees at Location 1 to enable comparison across years. All moss and lichen  
169 samples were placed in individual brown paper bags, which were stored in a cool, dry room to  
170 allow the samples to dehydrate. From each of the 2020 lichen samples, five subsamples of 0.25 g  
171 were placed in sterile 1.5 mL tubes and frozen at -20 °C for substrate DNA extraction. Table 1  
172 shows a summary of collected samples.

### 173 **2.2 Aseptic Technique**

174 16S rRNA gene amplicon sequencing studies focusing on low-biomass samples are prone  
175 to biases from external contamination during sample processing, DNA extraction, library  
176 preparation, and sequencing. Therefore, all subsequent tardigrade isolation and DNA extraction  
177 steps were carried out using barrier pipette tips (Axygen) and in a sterile work area dedicated to  
178 the project. A Bunsen burner was used to create a sterile field for all tardigrade isolations and  
179 DNA extractions.

### 180 **2.3 Tardigrade Extraction**

181 To extract tardigrades, each moss or lichen sample was soaked in glass-distilled water for  
182 a minimum of four hours. Subsamples of this water were then examined under a dissecting  
183 microscope, and tardigrades were extracted with Irwin loops (Schram and Davison 2012; Miller  
184 1997). The Irwin loop was disinfected by a flame between each collected tardigrade.

185 Next, isolated tardigrades were washed by immersion in droplets of PCR-grade water  
186 treated with diethyl pyrocarbonate (DEPC). Tardigrades were then transferred to a fresh drop of  
187 DEPC-treated water. This washing process was repeated for a total of three washes, and Irwin  
188 loops were sterilized between each wash. Three to six replicates of 30 tardigrades each were  
189 collected from each substrate sample (identified by replicate codes shown in Table 1) and were  
190 then stored in DEPC-treated water at -20 °C.

### 191 **2.4 DNA extraction and sequencing**

192 The DNeasy PowerLyzer PowerSoil Kit (Qiagen) was utilized for DNA extraction.  
193 Substrate samples were first ground with a sterilized pestle before being transferred to the bead  
194 tubes, while tardigrades were directly transferred to bead tubes. Bead tubes were then transferred  
195 to a Bead Mill 24 homogenizer (Fisher Scientific). Tardigrades were homogenized using a single  
196 30 second cycle at 5.00 speed, and substrate samples were homogenized using three 30 second  
197 cycles with 10 seconds between cycles at 5.50 speed. Homogenized bead tubes were then

198 centrifuged at 10,000 x g (30 seconds for tardigrades and 3 minutes for substrate) before  
199 proceeding according to the manufacturer's instructions; the optional 5 minute incubation at 2 -  
200 8°C was performed during steps 7 and 9. Following elution of DNA with 90 µL of elution buffer,  
201 DNA quality and concentration of a 1 µL sample was measured using a NanoDrop spectrometer.  
202 Extracted DNA was stored at -20°C.

203 DNA was loaded onto sterile 96 well plates for library preparation and sequencing. In  
204 addition to the tardigrade and substrate samples, three types of controls were included to account  
205 for contamination. Six tardigrade processing controls (TPC, equivalent to RIDE sampling blank  
206 controls) were created by applying the tardigrade extraction and subsequent DNA extraction  
207 protocols to blank samples. Ten DNA processing controls (DPC, equivalent to RIDE DNA  
208 extraction blank controls) were created by conducting DNA extraction on 100 µL of DEPC-  
209 treated water. Finally, ten wells were loaded with DEPC-treated water to form the library  
210 processing controls (LPC, equivalent to RIDE no-template amplification controls). Controls and  
211 samples were then submitted for library prep and 16S rRNA gene amplicon sequencing targeting  
212 the V4 region at the Iowa State University DNA facility. Library preparation was conducted  
213 following the Earth Microbiome Project 16S Illumina amplicon protocol  
214 (<https://earthmicrobiome.org/protocols-and-standards/16s/>) with the following modifications: (1)  
215 a single amplification was conducted for each sample rather than in triplicate, (2) PCR  
216 purification was conducted using the QIAquick PCR Purification Kit (Qiagen), and (3) all  
217 reactions and purification steps were conducted at half volume using a Mantis liquid handler  
218 (Formulamatrix) which was cleaned with isopropanol prior to library preparation. Libraries were  
219 loaded onto the MiSeq platform at a concentration of ~ 4pM, and paired-end sequencing was  
220 conducted at 500 cycles.

## 221 **2.5 Data Analysis**

222 Following sequencing, three paired end samples representing replicates L6\_19\_Tr2\_li2,  
223 L6\_19\_Tr4\_li1, and L5\_19\_Tr3\_li3 (Table 1) were removed from the dataset due to poor  
224 quality. Raw reads were processed with mothur version 1.43.0. Sequences were screened to  
225 remove reads that contained any ambiguities, were shorter than 252 bases, and had  
226 homopolymeric sequences greater than eight bases. In total, 1,157,089 reads were removed from  
227 the raw dataset of 7,805,248 reads. Screened reads were then aligned against the SILVA  
228 alignment version 138, and reads which aligned outside the region covered by 95% of the  
229 alignment were removed. The SILVA database was also used to remove 145,663 chimeric  
230 sequences and to classify remaining sequences. *De novo* OTU clustering was then conducted at a  
231 99% similarity threshold.

232 R version 4.0.3 running packages decontam (Davis et al. 2018), phyloseq (McMurdie and  
233 Holmes 2013), and corncob (Martin, Witten, and Willis 2020), as well as a more efficient  
234 implementation of DivNet known as divnet-rs (<https://github.com/mooreryan/divnet-rs>) running  
235 in Rust, were used for subsequent analyses. Using decontam, contaminant OTUs were identified  
236 and removed based on their relative prevalence in control vs. true samples (prevalence method,  
237 threshold 0.25) (Davis et al. 2018). Next, OTUs with fewer than 10 reads in experimental  
238 samples were removed. From these data, alpha diversity parameters (Shannon and Simpson)  
239 were calculated using DivNet and divnet-rs (Willis and Martin 2020). Relative abundance,

240 differential abundance, and differential variability of taxa were calculated in corncob using a  
241 beta-binomial model (Martin, Witten, and Willis 2020). Differences were declared significant  
242 when False Discovery Rate (FDR)-corrected  $P$  values (Benjamini and Hochberg 1995) were less  
243 than 0.05. Principal Coordinates Analysis (PCoA) was conducted in phyloseq using the default  
244 Bray-Curtis distance.

## 245 **2.6 Identification of unclassified putative plant pathogens and endosymbionts**

246 In the cases where OTUs of interest were not classified by mothur to the genus level,  
247 BLAST and RDP Classifier (Camacho et al. 2009; Wang et al. 2007) results were used to  
248 provide additional information about taxonomic classification. First, the mothur command  
249 “get.oturep” was used to generate a FASTA file containing the representative sequence for each  
250 OTU; OTUs with fewer than 10 reads in experimental samples were removed. BLAST analysis  
251 was performed using BLAST+ v2.11.0. The NCBI 16S RefSeq collection (representing 22,061  
252 taxa) (O’Leary et al. 2016) was downloaded and converted into a BLAST database using the  
253 “makeblastdb” command. The “blastn” command was then run against this database using the  
254 representative sequence FASTA as the query. Results with the 15 lowest E-values were kept for  
255 each OTU. The representative sequence FASTA was also entered into the RDP Classifier web  
256 tool version 2.11 using 16S rRNA training set 18  
257 (<https://rdp.cme.msu.edu/classifier/classifier.jsp>), and the assignment detail for all OTUs was  
258 downloaded.

## 259 **2.7 Data and Code Availability**

260 Raw sequencing files are deposited at the Sequence Read Archive. Mothur output and  
261 code used for analysis is available at [https://github.com/LTibbs/tardigrade\\_microbiome](https://github.com/LTibbs/tardigrade_microbiome).

## 262 **3 Results**

263 In total, 118 DNA samples and 26 controls were sequenced. The DNA samples consisted  
264 of 20 from lichen as well as 89 and 9 from tardigrades extracted from lichen and moss,  
265 respectively, collected from a total of 23 different apple trees in six Iowa orchards. The controls  
266 consisted of 6 TPCs, 10 DPCs, and 10 LPCs. From these sequences, 248,493 OTUs were  
267 identified by mothur. The decontam package identified and removed 986 OTUs as contaminants.  
268 Of the remaining OTUs, 235,652 were removed because they were represented by fewer than ten  
269 reads in the experimental samples, leaving 11,855 OTUs for further analysis.

270 Mothur classification and decontam scores for all OTUs with more than 10 reads in  
271 experimental samples are shown in Table S1. BLAST results and RDP Classifier results for these  
272 OTUs can be found in Tables S2 and S3, respectively. Relative abundance of OTUs by sample  
273 and by contrast are provided in Table S4 and Tables S5-S8, respectively; significantly  
274 differentially abundant and variable phyla, genera, and OTUs across contrast levels are presented  
275 in Table S9. Overall, the five most abundant phyla were *Proteobacteria*, *Bacteroidota*,  
276 *Actinobacteriota*, *Firmicutes*, and *Acidobacteriota* (Fig. S2), while the three most abundant  
277 genera were *Pseudomonas*, *Bradyrhizobium*, and an unclassified *Enterobacteriaceae* (Fig. S3).  
278 From the PCoA of all samples, the first principal coordinate clearly separates substrate samples

279 from tardigrade samples, while the second coordinate tends to separate the 2020 from the 2019  
280 samples. Samples from different locations and from moss and lichen are not clearly separated by  
281 the first two coordinates (Fig. S4).

### 282 **3.1 Contrast 1: Location**

283 The tardigrade community microbiome differed significantly across locations, as shown  
284 by the Simpson and Shannon indices, which differed significantly in most pairwise comparisons  
285 of locations (Table 2). Across locations, 13 phyla and 44 genera were both significantly  
286 differentially abundant and significantly differentially variable. Sixteen OTUs were significantly  
287 differentially abundant only, four OTUs were significantly differentially variable only, and three  
288 OTUs were both significantly differentially abundant and variable (Table S9). These identified  
289 differential taxa included the aforementioned top five phyla (*Proteobacteria*, *Bacteroidota*,  
290 *Actinobacteriota*, *Firmicutes*, and *Acidobacteriota*) and top three genera (*Pseudomonas*,  
291 *Bradyrhizobium*, and unclassified *Enterobacteriaceae*) from the experiment as a whole. Despite  
292 these differences, the locations clustered together in the PCoA (Fig. 1).

### 293 **3.2 Contrast 2: Moss vs. Lichen**

294 The community microbiome of tardigrades extracted from moss did not differ  
295 significantly in alpha diversity from that of tardigrades extracted from lichen as measured by the  
296 Shannon and Simpson indices (Table 2). PCoA further demonstrates that the overall microbial  
297 community did not differ by substrate type (Fig. 1). However, between tardigrades collected  
298 from moss and those from lichen, five phyla and 11 genera were both significantly differentially  
299 abundant and significantly differentially variable, while three OTUs were significantly  
300 differentially abundant only (Table S9). These included the common phyla *Firmicutes* and  
301 *Bacteroidota*; of these, *Firmicutes* were more abundant in moss-associated and *Bacteroidota* in  
302 lichen-associated tardigrades (Table S10, Fig. 2).

303

### 304 **3.3 Contrast 3: Tardigrades vs. Substrate**

305 The microbiota of tardigrades was significantly less diverse than that of their lichen  
306 substrate, as measured by both Shannon and Simpson indices (Table 2); the tardigrade and  
307 substrate samples also formed distinct clusters as shown by PCoA (Fig. 1). Between tardigrades  
308 and their substrate, 17 phyla, 181 genera, and 101 OTUs were significantly differentially  
309 abundant and variable, while 308 OTUs were significantly differentially abundant only and 124  
310 OTUs were significantly differentially variable only (Table S9). These differential taxa included  
311 four of the top five phyla (all except *Proteobacteria*) and all three of the three most abundant  
312 genera from the experiment as a whole. Remarkably, the relative abundance of *Firmicutes* was  
313 nearly a thousand times higher in the tardigrades (20.5%) than in their substrate (0.021%) (Table  
314 S10, Fig. 2).

315



### 316 **3.4 Contrast 4: Year**

317 From 2019 to 2020, the tardigrade community microbiome increased in diversity as  
318 measured by the Simpson index, though no significant difference was found between the  
319 Shannon indices (Table 2). The two years also formed mostly distinct clusters in the PCoA (Fig.  
320 1). Between the two years, 44 genera and one OTU were significantly differentially variable and  
321 abundant, while two phyla and 26 OTUs were significantly differentially abundant only (Table  
322 S9). These differential taxa included two of the five most common phyla (*Proteobacteria* and  
323 *Actinobacteriota*) and two of the three most common genera (*Pseudomonas* and unclassified  
324 *Enterobacteriaceae*). The unclassified *Enterobacteriaceae* had a particularly large change in  
325 relative abundance, decreasing more than 160-fold from 10.1% in 2019 to 0.062% in 2020  
326 (Table S11, Fig. 3).

## 327 **4 Discussion**

### 328 **4.1 Tardigrade Community**

329 This study examined the microbiota of the full tardigrade community from a particular  
330 substrate sample, in contrast to previous surveys that studied isolated species, often from  
331 laboratory cultures (Vecchi et al. 2018; Kaczmarek et al. 2020; Mioduchowska et al. 2021;  
332 Zawierucha et al. 2022). It is of course desirable to identify species-specific microbiota, as  
333 Vecchi et al. (2018) found that tardigrade-associated bacteria varied among tardigrade species.  
334 However, while it is occasionally possible in samples containing only a few tardigrade species to  
335 extract members of each for study (Vecchi et al. 2018), many environmental samples contain  
336 numerous species, including cryptic species that may be difficult or impossible to distinguish  
337 without molecular, life cycle, or other data (Roberto Guidetti et al. 2016; Cesari et al. 2013). For  
338 example, in the current survey, at least three tardigrade genera (*Milnesium*, *Ramazzottius*, and  
339 *Paramacrobotus*) were observed, but all would require additional morphometric or egg  
340 observations to identify to species level (Michalczyk et al. 2012; R. Guidetti et al. 2009; Kinchin  
341 1996; Binda 1987). Consequently, laboratory culturing is usually required for identification of  
342 tardigrade species, but Vecchi et al. (2018) found that culturing significantly affects the  
343 tardigrade microbiome. Therefore, the current study has the unique advantage of better reflecting  
344 the tardigrade community microbiome in its natural state compared to studies that focus on  
345 cultured tardigrades.

346 While tardigrade species were not identified in this study, a December 2015 collection  
347 effort at Location 1 provides information on tardigrade diversity in the area. From lichen  
348 growing on some of the same apple trees used in the current study, the previous study identified  
349 *Milnesium* cf. *barbadosense* Meyer and Hinton 2012; *Mil. burgessi* Schlabach, Donaldson,  
350 Hobelman, Miller, and Lowman, 2018; *Mil. swansoni* Young, Chappell, Miller, and Lowman,  
351 2016; and *Pam. (A.) tonollii* (Ramazzotti, 1956), as well as members of *Milnesium* Doyère,  
352 1840; *Ramazzottius* Binda and Pilato, 1986; *Paramacrobotus* Guidetti, Schill, Bertolani,  
353 Dandekar and Wolf, 2009; and Macrobiotidae Thulin, 1928 not identifiable to species (Tibbs-  
354 Cortes, Tibbs-Cortes, and Miller 2020). While tardigrade communities are dynamic across both  
355 time (Schuster and Greven 2007, 2013) and space (Meyer 2008, 2006), the dominant species

356 present in tardigrade communities in a given area can remain remarkably stable across years  
357 (Schuster and Greven 2007; Nelson and McGlothlin 1996). This suggests that species  
358 information from the 2015 survey may be relevant to the current study.

359

## 360 **4.2 Reducing Effects of Contamination**

361 All tardigrade microbiome surveys, including the current study, employed laboratory  
362 technique to reduce contamination by washing the tardigrades in sterile water before DNA  
363 extraction (Vecchi et al. 2018; Mioduchowska et al. 2021; Kaczmarek et al. 2020; Zawierucha et  
364 al. 2022). Working in a sterile environment further decreases contamination; therefore,  
365 Zawierucha *et al.* extracted tardigrades from substrate in a sterile environment (laminar flow  
366 chamber) (2022), and we worked in a sterile field created by a Bunsen burner throughout the  
367 experiment. Three previous studies included one type each of negative controls recommended by  
368 the RIDE standards for low biomass studies (Eisenhofer et al. 2019) (DNA extraction blank in  
369 (Mioduchowska et al. 2021), sampling blank in (Kaczmarek et al. 2020), and no-template  
370 amplification control in (Zawierucha et al. 2022)). Kaczmarek *et al.* and Mioduchowska *et al.*  
371 did not sequence the negative controls; instead, they performed PCR amplification of these  
372 controls and determined that no contamination was present because no bands were visible  
373 (Kaczmarek et al. 2020; Mioduchowska et al. 2021). However, samples without visible bands  
374 from PCR can generate sequencing reads (Davis et al. 2018) and would not detect contaminants  
375 introduced during library preparation or sequencing steps. Zawierucha *et al.* removed all OTUs  
376 present in the no-template amplification control from analysis (2022). However, low levels of  
377 true sequences, especially from high-abundance OTUs, are often present in negative controls due  
378 to cross-contamination of samples; these biologically important OTUs would therefore be  
379 removed from the analysis (Davis et al. 2018; Karstens et al. 2019). In our study, we included  
380 and sequenced all three RIDE-recommended types of negative controls.

381 No previous survey of the tardigrade microbiota has employed model-based *in silico*  
382 contaminant identification and removal, which we accomplished using the decontam package.  
383 Decontam removed 986 OTUs as contaminants, including five that would otherwise have been in  
384 the top ten OTUs in the study by read count. Of course, further improvements are always  
385 possible. Contaminants are expected to differ in prevalence among negative control types  
386 depending on their point of introduction, but current *in silico* contamination removal methods  
387 treat all negative controls identically (Davis et al. 2018). Future development of a method that  
388 leverages the unique information provided by each type of negative control would therefore be  
389 desirable. However, by working under a flame, including and sequencing all recommended types  
390 of negative controls, and leveraging *in silico* contaminant identification and removal, we have  
391 produced what we expect to be the tardigrade microbiome survey least affected by contamination  
392 to date.

## 393 **4.3 Tardigrade Community Microbiome by Contrast**

394 We investigated the tardigrade community microbiome across four contrasts. First, we  
395 determined that the tardigrade community microbiome varied significantly in structure across  
396 locations (Table 2). Vecchi *et al.* (2018) found that an average of 15.4% of the microbial OTUs

397 in a tardigrade collected from moss or lichen originate from its substrate. Therefore, known  
398 impacts of geographical location on microbial communities (Baldrian 2017; Coller et al. 2019)  
399 could have resulted in different microbial communities present in each location to inoculate the  
400 tardigrades. Additionally, as the tardigrade microbiota is species-specific (Vecchi et al. 2018),  
401 the differences in microbial communities observed across locations may reflect spatial variation  
402 in tardigrade communities' species composition (Meyer 2008). Of course, these explanations are  
403 not mutually exclusive and could both play a role in shaping distinct tardigrade community  
404 microbiomes across locations. Vecchi *et al.* surveyed tardigrades of the same species collected  
405 from different locations, but they did not test for differences in the microbiome across locations.  
406 However, they did identify an OTU in the genus *Luteolibacter* that was significantly associated  
407 with *Ram. oberhaeuseri* collected from a location at 34 meters above sea level but not from  
408 another location 797 meters above sea level (Vecchi et al. 2018), suggesting that future work  
409 may detect differences in the microbiota of the same tardigrade species across locations.

410 In contrast two, the community microbiome of tardigrades collected from lichen was  
411 compared with that of tardigrades collected from moss on the same trees. While a few taxa were  
412 significantly differentially abundant and variable across substrates, the two substrates did not  
413 differ in alpha diversity and were not separated by PCoA (Fig. 1). This similarity in the  
414 tardigrade community microbiome was initially surprising, as previous literature has  
415 demonstrated significant differences between the microbiota of moss and lichen even on the  
416 same tree (Aschenbrenner et al. 2017). However, this similarity across substrates could be due to  
417 the presence of similar tardigrade species, as previous studies have failed to demonstrate  
418 significant differences between tardigrade communities found in moss and lichen (Young and  
419 Clifton 2015; Nelson, Bartels, and Fegley 2020). In future surveys, it would be interesting to  
420 compare the microbiota of tardigrades from additional substrate types (e.g., soil) and to  
421 determine if this similarity in tardigrade microbiome across substrates persists at the species as  
422 well as the community level.

423 Results of contrast three demonstrate that the tardigrade community microbiome is  
424 distinct from and significantly less diverse than that of its lichen substrate (Table 2, Fig. 1). This  
425 result agrees with previous studies that found relatively higher diversity in substrates than in their  
426 resident Ecdysozoans, including wild tardigrades collected from moss and lichen (Vecchi et al.  
427 2018), cultured tardigrades (Kaczmarek et al. 2020), and the nematodes *Meloidogyne hapla*  
428 (Adam et al. 2014) and *Caenorhabditis elegans* (Johnke, Dirksen, and Schulenburg 2020). This  
429 study is the first to demonstrate this trend at the tardigrade community rather than species level.  
430 Vecchi et al. (2018) suggested that the lower microbial diversity in tardigrades with respect to  
431 their substrates may be due to the small size of tardigrades limiting the biomass and therefore the  
432 diversity of their microbiome (small host hypothesis) and/or to selectiveness of tardigrades  
433 inhibiting growth of some bacterial species and promoting growth of others (selective host  
434 hypothesis). Supporting the selective host hypothesis, earlier work found that tardigrades could  
435 be successfully inoculated with some bacteria (*Xanthomonas*) but not others (*Serratia*) (Krantz,  
436 Benoit, and Beasley 1999). It is possible that some bacteria have co-evolved with tardigrades,  
437 becoming permanent residents of the gastrointestinal tract or cuticle, a hypothesis that has been  
438 suggested for the Ecdysozoan *C. elegans* (F. Zhang et al. 2017). The life cycle of tardigrades  
439 poses a unique selective pressure on any permanent residents of the microbiota, as these

440 organisms would also have to survive within the tardigrade during cryptobiosis. This would be  
441 especially true for the obligate endosymbiotic taxa *Rickettsiales* and *Polynucleobacter* previously  
442 observed in tardigrades (Vecchi et al. 2018; Roberto Guidetti et al. 2020; Kaczmarek et al. 2020;  
443 Mioduchowska et al. 2021), as well as for the *Rickettsia* identified in the current study (see  
444 below).

445 Contrast four determined that the tardigrade community microbiome is temporally  
446 dynamic, changing significantly on the same trees from 2019 to 2020 (Table 2, Fig. 1). Again,  
447 this may be due to changes in habitat microbiome, as microbiota of other substrates (e.g., soil  
448 and litter) are known to vary across years due to changing environmental factors such as nutrient  
449 availability (Martinović et al. 2021). This variation may also be due to temporal changes in the  
450 tardigrade community composition; although tardigrade species present may remain consistent in  
451 a location over years, their relative abundances shift in part due to changes in rainfall, humidity,  
452 and temperature (Schuster and Greven 2007). This temporal variability raises important  
453 implications for future studies of the tardigrade community microbiome. For example, the  
454 relative abundance of putative phytopathogens differed significantly across years (Table S9).  
455 Future work could identify temporal variables affecting the ability of tardigrades to act as  
456 potential reservoirs of phytopathogens and other bacteria. We also encourage further studies of  
457 the tardigrade microbiome to account for temporal changes and to investigate this variation with  
458 additional time points to increase resolution.

#### 459 **4.4 Tardigrade-Associated Taxa**

460 In this study, the five most abundant phyla were *Proteobacteria*, *Firmicutes*,  
461 *Bacteroidota*, *Actinobacteria*, and *Acidobacteria* (Table S10). All of these except *Acidobacteria*  
462 were previously reported as highly abundant in at least two of the three previous tardigrade  
463 microbiome surveys that presented results at a phylum level, with *Proteobacteria* identified as  
464 the most abundant phylum in all cases (Vecchi et al. 2018; Kaczmarek et al. 2020;  
465 Mioduchowska et al. 2021). Combined, the tardigrades in these studies represent a diverse set of  
466 species, including wild and laboratory-reared specimens isolated from multiple continents,  
467 suggesting that the predominance of these phyla is broadly characteristic of the microbiome of  
468 Tardigrada, regardless of species or location. These phyla, especially *Proteobacteria*, are also  
469 dominant in the microbiomes of other Ecdysozoans, including soil nematodes (Dirksen et al.  
470 2016; Elhady et al. 2017; Adam et al. 2014), marine nematodes (Arcos et al. 2021), and insects  
471 (Colman, Toolson, and Takacs-Vesbach 2012; Engel and Moran 2013). The tardigrade  
472 microbiota therefore appears similar to that of other Ecdysozoans at the phylum level.

473 A number of OTUs significantly more abundant in tardigrades than in their substrate in  
474 this study belong to taxa previously identified in the tardigrade microbiome. These include  
475 members of *Enhydrobacter* (Vecchi et al. 2018), *Enterobacteriaceae* (Mioduchowska et al.  
476 2021; Kaczmarek et al. 2020), and *Acinetobacter* (Vecchi et al. 2018; Mioduchowska et al. 2021)  
477 (Table S9). In this study, OTU 22 was classified as *Enhydrobacter*, and its abundance in the  
478 tardigrade population varied over time, increasing significantly from 2019 (0.21%) to 2020  
479 (2.1%) (Table S8, Table S9). It is possible that *Enhydrobacter* is common to Ecdysozoan  
480 microbiomes, as it is also an abundant taxon in the gut contents of larval wood wasps (J. Li et al.  
481 2021) and nematodes (Adam et al. 2014). Members of *Enterobacteriaceae* included OTUs 2 and

482 20. OTU 20 was further identified as a member of the *Escherichia/Shigella* complex, but OTU 2  
483 could not be classified to the genus level (Table S2, Table S3). OTU 2 showed significant  
484 temporal variation, decreasing in relative abundance from 10.0% to 0.062% from 2019 to 2020  
485 (Table S8, Table S9). *Enterobacteriaceae* is also highly represented in the gut microbiota of  
486 insects (Moro et al. 2021; Hernández-García et al. 2017) and nematodes (Zhou et al. 2022;  
487 Zimmermann et al. 2020). This suggests that *Enterobacteriaceae* may be residents of the  
488 tardigrade digestive tract. Finally, OTU 16 was a member of *Acinetobacter* that increased  
489 significantly in abundance from 2019 (0.000052%) to 2020 (2.3%) and was one of the three  
490 OTUs significantly differentially abundant across substrate type (Table S8, Table S9).  
491 *Acinetobacter* is associated with the cuticle of the nematodes *M. hapla*, *M. incognita*, and  
492 *Pratylenchus penetrans* (Adam et al. 2014; Elhady et al. 2017), suggesting that it may also be  
493 associated with the cuticle of tardigrades.

494 However, many of the tardigrade-associated taxa observed in this study have not been  
495 previously reported in the tardigrade microbiome. In fact, the most abundant OTU across all  
496 samples in this study (OTU 1) was a member of the genus *Bradyrhizobium*, which was not  
497 previously reported from the tardigrade microbiome. This OTU was also spatially dynamic,  
498 differing significantly in abundance across locations (Table S9). *Bradyrhizobium* has been  
499 previously observed in the microbiota of plant pathogenic nematodes (Eberlein et al. 2016;  
500 Adam et al. 2014) and leaf hoppers (Horgan et al. 2019). This genus has also been found in the  
501 lichen microbiome (Bates et al. 2011; Erlacher et al. 2015; Graham et al. 2018), perhaps  
502 indicating that tardigrades acquire this bacterium from their habitat. Another tardigrade-  
503 associated genus, *Micrococcus*, was differentially abundant across both locations and years  
504 (Table S9). This genus has been reported from the cuticles of soil nematodes (Adam et al. 2014)  
505 as well as fish parasitic nematodes (Arcos et al. 2021), suggesting that *Micrococcus* may be  
506 associated with the tardigrade cuticle. Another notable tardigrade-associated genus in this study  
507 was *Nakamurella*, represented primarily by OTU 33, which showed differential abundance  
508 across locations (Table S9). *Nakamurella intestinalis* has been isolated from the feces of another  
509 Ecdysozoan, the katydid *Pseudorynchus japonicus* (Kim et al. 2017). *N. endophytica* and *N.*  
510 *flava* were identified as endophytes of mangroves and mint, respectively (Yan et al. 2020; Tuo et  
511 al. 2016), and *N. albus* and *N. leprariae* were originally discovered in lichens (Jiang et al. 2020;  
512 An et al. 2021). This suggests that tardigrades could obtain endophytic or lichen-dwelling  
513 *Nakamurella* from their habitat.

#### 514 **4.5 Putative Endosymbionts**

515 Our survey corroborates previous observations of putative endosymbionts of the obligate  
516 intracellular order *Rickettsiales* associated with tardigrades. Three of the four previous surveys of  
517 the tardigrade microbiome have detected OTUs of this order (Vecchi et al. 2018; Roberto  
518 Guidetti et al. 2020; Kaczmarek et al. 2020; Mioduchowska et al. 2021); in addition to  
519 unclassified *Rickettsiales*, these OTUs included members of *Wolbachia* (Mioduchowska et al.  
520 2021), *Anaplasmatataceae*, and *Ca. Tenuibacteraceae* (Roberto Guidetti et al. 2020). Kaczmarek  
521 et al. also detected the obligate intracellular genus *Polynucleobacter* (2020). In the current  
522 survey, we identified two *Rickettsiales* OTUs. Of these, one was classified by mothur as  
523 *Wolbachia* (OTU 3606), and the other was further classified as *Rickettsia* (OTU 180) by BLAST

524 and RDP analysis (Table S2, Table S3). The relative abundance of OTU 180 was significantly  
525 higher in tardigrades (0.88%) than in their lichen substrate (0.0012%) (Table S7) as well as  
526 significantly higher in 2020 (1.0%) than in 2019 (0.00026%) (Table S8, Table S9). OTU 3606  
527 was numerically more abundant in tardigrades (0.030%) than substrate (0.0000000000051%),  
528 though this difference was not statistically significant (Table S7). Taken together, the  
529 intracellular nature of *Rickettsiales* and the higher abundance in tardigrades suggests that OTUs  
530 180 and 3606 are endosymbionts of tardigrades.

531 The presence of endosymbionts may have implications for tardigrade reproduction and  
532 evolution, as members of *Rickettsia* and *Wolbachia* are known to manipulate host reproduction  
533 in other Ecdysozoans. *Wolbachia* is well-known for causing parthenogenesis in nematodes and  
534 arthropods, as well as feminization of males, cytoplasmic incompatibility, and male-killing  
535 (Werren, Baldo, and Clark 2008; Kraaijeveld et al. 2011; Correa and Ballard 2016; Kajtoch and  
536 Kotásková 2018). Similarly, *Rickettsia* can induce parthenogenesis (Hagimori et al. 2006;  
537 Giorgini et al. 2010) and male-killing (Lawson et al. 2001) in arthropods. Parthenogenesis is  
538 common in tardigrades (Bertolani 2001; Guil et al. 2022). Further investigation is necessary to  
539 determine if this is due to reproductive manipulators such as *Rickettsia* and *Wolbachia*. Future  
540 analysis could follow the example of Guidetti *et al.* by incorporating FISH to confirm the  
541 presence of these and other endosymbionts within tardigrade tissues (2020).

542

#### 543 **4.6 Putative Phytopathogens**

544 Our analysis also aimed to determine whether wild tardigrades living in apple orchards  
545 harbor phytopathogenic bacteria, and in fact, the second most abundant genus overall found in  
546 this survey was *Pseudomonas*, which contains more than twenty known plant pathogens (Höfte  
547 and De Vos 2006). *Pseudomonas* was significantly associated with tardigrades (relative  
548 abundance in tardigrades and substrate of 2.7% and 0.051%, respectively) (Table S11) and was  
549 spatially and temporally dynamic in the tardigrade community microbiome, as relative  
550 abundance of *Pseudomonas* decreased significantly from 2019 (19.6%) to 2020 (3.0%) and  
551 differed significantly across locations (Table S9, Table S11). *Pseudomonas* was also detected in  
552 all four of the previous surveys of the tardigrade microbiome (Vecchi et al. 2018; Mioduchowska  
553 et al. 2021; Kaczmarek et al. 2020; Zawierucha et al. 2022), and Vecchi *et al.* identified it as part  
554 of the core tardigrade microbiome (2018). *Pseudomonas* is also present in the microbiota of soil  
555 nematodes (Adam et al. 2014; Dirksen et al. 2016; Zimmermann et al. 2020) and insects  
556 (Hernández-García et al. 2017; Horgan et al. 2019; Xue et al. 2021). Notably, other Ecdysozoans  
557 (insects) act as vectors of *P. syringae* (Stavrínides, McCloskey, and Ochman 2009; Donati et al.  
558 2017), which is one of the most agriculturally damaging *Pseudomonas* species (Höfte and De  
559 Vos 2006; Xin, Kvitko, and He 2018). However, *Pseudomonas* is very diverse, containing many  
560 non-pathogenic species (Silby et al. 2011; Passera et al. 2019). In fact, some *Pseudomonas*  
561 isolates from wild *C. elegans* confer resistance to fungal pathogens in their hosts (Dirksen et al.  
562 2016), raising the possibility that *Pseudomonas* could be similarly beneficial to tardigrades.

563 Two additional putative phytopathogens were significantly more abundant in tardigrades  
564 than their substrate. The first, OTU 261, was identified by mothur as a member of *Ralstonia*, a  
565 genus containing the phytopathogenic *R. solanacearum* complex. In addition to being found at

566 significantly higher abundance in tardigrades (0.018%) than their substrate (.00017%) (Table  
567 S7), OTU 261 was temporally dynamic, decreasing significantly from 2019 (0.16%) to 2020  
568 (0.00068%) (Table S8, Table S9). *Ralstonia* has been previously observed in the tardigrade *Pam.*  
569 *fairbanksi* (Mioduchowska et al. 2021) and in nematodes (Elhady et al. 2017; Eberlein et al.  
570 2016). The *R. solanacearum* complex causes major yield losses in food crops including  
571 tomatoes, bananas, and potatoes (Yuliar, Nion, and Toyota 2015; Paudel et al. 2020). Two  
572 notable members of this complex are spread by insect vectors; the cercopoids *Hindola fulva* and  
573 *H. strata* act as vectors of *R. syzygii*, while the Blood Disease Bacterium is spread  
574 nonspecifically by pollinators (Eden-Green et al. 1992; Remenant et al. 2011).

575 The second, OTU 208, was classified by BLAST and RDP analysis to the  
576 *Erwinia/Pantoea* cluster (Table S2 Table S3), which includes a number of economically  
577 important phytopathogens (Kido et al. 2008; Y. Zhang and Qiu 2015; Dutkiewicz et al. 2016;  
578 Shapiro et al. 2016). *E. amylovora* is of particular note as it causes fire blight in apple trees  
579 (Aćimović et al. 2015). This OTU had a significantly higher relative abundance of 0.046% in  
580 tardigrades compared to 0.0044% in their substrate (Table S7, Table S9). While neither *Erwinia*  
581 nor *Pantoea* have previously been identified in tardigrades, *Erwinia* has been found in  
582 arthropods (Xue et al. 2021) and nematodes (Eberlein et al. 2016). Additionally, multiple  
583 phytopathogens in *Pantoea* and *Erwinia* are transmitted by insect vectors (Dutkiewicz et al.  
584 2016; Ordax et al. 2015; Sasu et al. 2010; Basset et al. 2000; Walterson and Stavrinos 2015).  
585 However, it is also possible that OTU 208 represents a symbiont in tardigrades, as *Erwinia* also  
586 includes the olive fly obligate gut symbiont *Candidatus Erwinia dacicola* (Blow et al. 2020).

587 Additional putative plant pathogens were observed at lower abundances in the tardigrade  
588 community microbiome and were not significantly more abundant in tardigrades than their  
589 substrate. These include another *Ralstonia* (OTU 1556) and OTU 1620, which was classified as  
590 *Pectobacterium* by BLAST and RDP (Table S2, Table S3). Members of *Pectobacterium* cause  
591 soft rot diseases in economically important plants, and some strains are capable of infecting  
592 multiple plant species (Ma et al. 2007; X. Li et al. 2020). Additionally, prompted by previous  
593 observation of the tardigrade *Mac. hufelandi* acting as a vector of the plant pathogen  
594 *Xanthomonas campestris* (Benoit et al. 2000), we searched the tardigrade community  
595 microbiome for members of *Xanthomonas*. OTUs 10,409 and 12,281 were classified as  
596 *Xanthomonas* (OTU 10,409 by BLAST and RDP analysis), but were both at extremely low  
597 abundance (Table S4).

598 In summary, we observed the presence of multiple putative plant pathogens in the  
599 community microbiome of tardigrades isolated from apple orchards. Tardigrades could act as  
600 vectors or reservoirs of these putative pathogens, a possibility raised by the previous observation  
601 of *Mac. hufelandi* as a vector of *X. campestris* (Benoit et al. 2000). However, a major limitation  
602 of this study is the use of only 16S rRNA amplicon sequencing. Because multiple marker genes  
603 are required to distinguish among species within *Pseudomonas*, *Ralstonia*, *Erwinia*, and *Pantoea*  
604 (Y. Zhang and Qiu 2015; Paudel et al. 2020; Palmer et al. 2017; Gomila et al. 2015; Saati-  
605 Santamaría et al. 2021), we were unable to identify OTUs in our study to species level.  
606 Therefore, we are unable to determine whether the identified OTUs in plant pathogenic genera  
607 are themselves phytopathogens. We encourage future analyses of tardigrade-associated bacteria

608 in these groups through techniques such as metagenome sequencing and multilocus sequence  
609 typing to clarify this point.

## 610 **5 Conclusion**

611 This study is the first microbiome analysis of wild tardigrade populations in an  
612 agricultural setting and is also the first microbiome study assessing North American tardigrades.  
613 Our methods reduced the effects of contamination compared to other tardigrade microbiome  
614 studies by including aseptic technique, all three recommended control types, and *in silico*  
615 contaminant removal. We found that the tardigrade community microbiome is distinct from the  
616 substrate microbiota and varies across location and time. In addition to identifying putative  
617 endosymbionts, we also observed multiple tardigrade-associated taxa that may represent  
618 phytopathogens. The results of this study both increase our knowledge of the tardigrade  
619 microbiome and prompt new avenues of research.

## 620 **6 Author Contributions**

621 LTC: Conceptualization, Software, Formal Analysis, Investigation, Data Curation, Writing –  
622 Original Draft and Review & Editing, Visualization. BTC: Conceptualization, Investigation,  
623 Data Curation, Writing – Original Draft and Review & Editing, Visualization. SSE:  
624 Methodology, Resources, Writing - Review & Editing  
625

## 626 **7 Acknowledgements**

627 We wish to thank the owners of the studied apple orchards, including Dennise Smith, Roland  
628 Newby, Koenigs' Acres Farm, and others who wish to remain anonymous. We also thank Lucas  
629 Koester and Chiron Anderson for providing example phyloseq code and tutorials as well as  
630 useful input on the project. We thank the Iowa State University (ISU) DNA Facility for their  
631 input on sequencing, the ISU Department of Ecology, Evolution, and Organismal Biology for  
632 use of microscopes, the Iowa Geological Survey for shapefiles used to produce the collection  
633 map, and the ISU Plant Sciences Institute for funding. Laura and Bienvenido Tibbs-Cortes are  
634 supported by the National Science Foundation Graduate Research Fellowship Program (NSF  
635 GRFP Grant No. 1744592).  
636

## 637 **8 ORCID iDs**

638 Laura E. Tibbs-Cortes <https://orcid.org/0000-0003-3188-6820>  
639 Bienvenido W. Tibbs-Cortes <https://orcid.org/0000-0003-3435-4889>  
640 Stephan Schmitz-Esser <https://orcid.org/0000-0002-1907-0709>



641 **9**      **References**

- 642 Aćimović, Srđan G, Quan Zeng, Gayle C McGhee, George W Sundin, and John C Wise. 2015.  
643 “Control of Fire Blight (*Erwinia Amylovora*) on Apple Trees with Trunk-Injected Plant  
644 Resistance Inducers and Antibiotics and Assessment of Induction of Pathogenesis-Related  
645 Protein Genes.” *Frontiers in Plant Science* 6.  
646 <https://www.frontiersin.org/article/10.3389/fpls.2015.00016>.
- 647 Adam, Mohamed, Andreas Westphal, Johannes Hallmann, and Holger Heuer. 2014. “Specific  
648 Microbial Attachment to Root Knot Nematodes in Suppressive Soil.” *Applied and  
649 Environmental Microbiology* 80 (9): 2679–86. <https://doi.org/10.1128/AEM.03905-13>.
- 650 An, De-Feng, Shao-Juan Yang, Long-Qian Jiang, Xin-Yu Wang, Xiao-Yu Huang, Lei Lang,  
651 Xue-Mei Chen, et al. 2021. “*Nakamurella Leprariae* Sp. Nov., Isolated from a Lichen  
652 Sample.” *Archives of Microbiology* 204 (1): 19. [https://doi.org/10.1007/s00203-021-02626-](https://doi.org/10.1007/s00203-021-02626-7)  
653 [7](https://doi.org/10.1007/s00203-021-02626-7).
- 654 Arcos, Susana C, Felipe Lira, Lee Robertson, María Rosa González, Noelia Carballeda-Sangiao,  
655 Isabel Sánchez-Alonso, Laura Zamorano, et al. 2021. “Metagenomics Analysis Reveals an  
656 Extraordinary Inner Bacterial Diversity in Anisakids (Nematoda: Anisakidae) L3 Larvae.”  
657 *Microorganisms* 9 (5): 1088. <https://doi.org/10.3390/microorganisms9051088>.
- 658 Aschenbrenner, Ines Aline, Tomislav Cernava, Armin Erlacher, Gabriele Berg, and Martin  
659 Grube. 2017. “Differential Sharing and Distinct Co-Occurrence Networks among Spatially  
660 Close Bacterial Microbiota of Bark, Mosses and Lichens.” *Molecular Ecology* 26 (10):  
661 2826–38. <https://doi.org/https://doi.org/10.1111/mec.14070>.
- 662 Baldrian, Petr. 2017. “Forest Microbiome: Diversity, Complexity and Dynamics.” *FEMS  
663 Microbiology Reviews* 41 (2): 109–30. <https://doi.org/10.1093/femsre/fuw040>.
- 664 Basset, Alan, Ranjiv S Khush, Anne Braun, Louis Gardan, Frédéric Boccard, Jules A Hoffmann,  
665 and Bruno Lemaitre. 2000. “The Phytopathogenic Bacteria *Erwinia Carotovora* Infects  
666 *Drosophila* and Activates an Immune Response.” *Proceedings of the National Academy of  
667 Sciences* 97 (7): 3376. <https://doi.org/10.1073/pnas.97.7.3376>.
- 668 Bates, Scott T, Garrett W G Cropsey, J Gregory Caporaso, Rob Knight, and Noah Fierer. 2011.  
669 “Bacterial Communities Associated with the Lichen Symbiosis.” *Applied and  
670 Environmental Microbiology* 77 (4): 1309–14. <https://doi.org/10.1128/AEM.02257-10>.
- 671 Becquerel, Paul. 1950. “La Suspension de La Vie Au Dessous de 1/20 K Absolu Par  
672 Demagnetization Adiabatique de l’alun de Fer Dans Le Vide Les plus Elève.” *Comptes  
673 Rendus Des Séances de l’Académie Des Sciences* 231 (4): 261–63.
- 674 Benjamini, Yoav, and Yosef Hochberg. 1995. “Controlling the False Discovery Rate: A Practical  
675 and Powerful Approach to Multiple Testing.” *Journal of the Royal Statistical Society. Series  
676 B (Methodological)* 57 (1): 289–300. <http://www.jstor.com/stable/2346101>.
- 677 Benoit, Thomas G., Jennifer Locke, James R. Marks, and Clark W. Beasley. 2000. “Laboratory  
678 Transmission of *Xanthomonas Campestris* P.v. *Raphani* by a Tardigrade (Parachela =  
679 *Macrobiotidae*.” *Florida Entomologist* 83 (2): 197–99. <https://doi.org/10.2307/3496157>.
- 680 Bertolani, Roberto. 2001. “Evolution of the Reproductive Mechanisms in Tardigrades — A  
681 Review.” *Zoologischer Anzeiger - A Journal of Comparative Zoology* 240 (3): 247–52.  
682 <https://doi.org/https://doi.org/10.1078/0044-5231-00032>.
- 683 Binda, M.G. 1987. “*Ramazzottius*, Nuovo Genere Di Eutardigrado (Hypsibiidae).” *Animalia* 13  
684 (1986): 159–66.
- 685 Blow, Frances, Anastasia Gioti, Ian B Goodhead, Maria Kalyva, Anastasia Kampouraki, John

- 686 Vontas, and Alistair C Darby. 2020. “Functional Genomics of a Symbiotic Community:  
687 Shared Traits in the Olive Fruit Fly Gut Microbiota.” *Genome Biology and Evolution* 12  
688 (2): 3778–91. <https://doi.org/10.1093/gbe/evz258>.
- 689 Camacho, Christiam, George Coulouris, Vahram Avagyan, Ning Ma, Jason Papadopoulos,  
690 Kevin Bealer, and Thomas L Madden. 2009. “BLAST+: Architecture and Applications.”  
691 *BMC Bioinformatics* 10 (1): 421. <https://doi.org/10.1186/1471-2105-10-421>.
- 692 Cesari, Michele, Roberto Guidetti, Lorena Rebecchi, Ilaria Giovannini, and Roberto Bertolani.  
693 2013. “A DNA Barcoding Approach in the Study of Tardigrades.” *Journal of Limnology*.  
694 <https://doi.org/10.4081/jlimnol.2013.s1.e23>.
- 695 Coller, Emanuela, Alessandro Cestaro, Roberto Zanzotti, Daniela Bertoldi, Massimo Pindo,  
696 Simone Larger, Davide Albanese, Enzo Mescalchin, and Claudio Donati. 2019.  
697 “Microbiome of Vineyard Soils Is Shaped by Geography and Management.” *Microbiome* 7  
698 (1): 140. <https://doi.org/10.1186/s40168-019-0758-7>.
- 699 Colman, D R, E C Toolson, and C D Takacs-Vesbach. 2012. “Do Diet and Taxonomy Influence  
700 Insect Gut Bacterial Communities?” *Molecular Ecology* 21 (20): 5124–37.  
701 <https://doi.org/https://doi.org/10.1111/j.1365-294X.2012.05752.x>.
- 702 Correa, Claudia C, and J W O Ballard. 2016. “Wolbachia Associations with Insects: Winning or  
703 Losing Against a Master Manipulator.” *Frontiers in Ecology and Evolution* 3.  
704 <https://www.frontiersin.org/article/10.3389/fevo.2015.00153>.
- 705 Davis, Nicole M., Diana M. Proctor, Susan P. Holmes, David A. Relman, and Benjamin J.  
706 Callahan. 2018. “Simple Statistical Identification and Removal of Contaminant Sequences  
707 in Marker-Gene and Metagenomics Data.” *Microbiome* 6 (1): 1–14.  
708 <https://doi.org/10.1186/s40168-018-0605-2>.
- 709 Dirksen, Philipp, Sarah Arnaud Marsh, Ines Braker, Nele Heitland, Sophia Wagner, Rania  
710 Nakad, Sebastian Mader, et al. 2016. “The Native Microbiome of the Nematode  
711 *Caenorhabditis Elegans*: Gateway to a New Host-Microbiome Model.” *BMC Biology* 14  
712 (1): 38. <https://doi.org/10.1186/s12915-016-0258-1>.
- 713 Donati, Irene, Sofia Mauri, Giampaolo Buriani, Antonio Cellini, and Francesco Spinelli. 2017.  
714 “Role of *Metcalfa Pruinosa* as a Vector for *Pseudomonas Syringae* Pv. *Actinidiae*.” *The*  
715 *Plant Pathology Journal* 33 (6): 554–60. <https://doi.org/10.5423/PPJ.OA.04.2017.0074>.
- 716 Dutkiewicz, Jacek, Barbara Mackiewicz, Marta Kinga Lemieszek, Marcin Golec, and Janusz  
717 Milanowski. 2016. “*Pantoea Agglomerans*: A Mysterious Bacterium of Evil and Good. Part  
718 III. Deleterious Effects: Infections of Humans, Animals and Plants.” *Annals of Agricultural*  
719 *and Environmental Medicine* 23 (2): 197–205. <https://doi.org/10.5604/12321966.1203878>.
- 720 Dutta, B, A K Barman, R Srinivasan, U Avci, D E Ullman, D B Langston, and R D Gitaitis.  
721 2014. “Transmission of *Pantoea Ananatis* and *P. Agglomerans*, Causal Agents of Center  
722 Rot of Onion (*Allium Cepa*), by Onion Thrips (*Thrips Tabaci*) Through Feces.”  
723 *Phytopathology*® 104 (8): 812–19. <https://doi.org/10.1094/PHYTO-07-13-0199-R>.
- 724 Duveiller, E., C. Bragard, and H. Marite. 1997. “Bacterial Leaf Streak and Black Chaff Caused  
725 by *Xanthomonas Translucens*.” In *The Bacterial Disease of Wheat: Concepts and Methods*  
726 *of Disease Management*, 25–47.
- 727 Eberlein, Caroline, Holger Heuer, Stefan Vidal, and Andreas Westphal. 2016. “Microbial  
728 Communities in *Globodera Pallida* Females Raised in Potato Monoculture Soil.”  
729 *Phytopathology*® 106 (6): 581–90. <https://doi.org/10.1094/PHYTO-07-15-0180-R>.
- 730 Eden-Green, S J, R Balfas, T Sutarjo, and Jamalius. 1992. “Characteristics of the Transmission  
731 of Sumatra Disease of Cloves by Tube-Building Cercopoids, *Hindola* Spp.” *Plant*

- 732 *Pathology* 41 (6): 702–12. <https://doi.org/https://doi.org/10.1111/j.1365->  
733 3059.1992.tb02553.x.
- 734 Eisenhofer, Raphael, Jeremiah J. Minich, Clarisse Marotz, Alan Cooper, Rob Knight, and Laura  
735 S. Weyrich. 2019. “Contamination in Low Microbial Biomass Microbiome Studies: Issues  
736 and Recommendations.” *Trends in Microbiology* 27 (2): 105–17.  
737 <https://doi.org/10.1016/j.tim.2018.11.003>.
- 738 Elhady, Ahmed, Ariadna Giné, Olivera Topalovic, Samuel Jacquiod, Søren J Sørensen,  
739 Francisco Javier Sorribas, and Holger Heuer. 2017. “Microbiomes Associated with  
740 Infective Stages of Root-Knot and Lesion Nematodes in Soil.” *PLOS ONE* 12 (5):  
741 e0177145. <https://doi.org/10.1371/journal.pone.0177145>.
- 742 Engel, Philipp, and Nancy A Moran. 2013. “Functional and Evolutionary Insights into the  
743 Simple yet Specific Gut Microbiota of the Honey Bee from Metagenomic Analysis.” *Gut*  
744 *Microbes* 4 (1): 60–65. <https://doi.org/10.4161/gmic.22517>.
- 745 Erlacher, Armin, Tomislav Cernava, Massimiliano Cardinale, Jung Soh, Christoph W Sensen,  
746 Martin Grube, and Gabriele Berg. 2015. “Rhizobiales as Functional and Endosymbiotic  
747 Members in the Lichen Symbiosis of *Lobaria Pulmonaria* L.” *Frontiers in Microbiology* 6:  
748 53. <https://doi.org/10.3389/fmicb.2015.00053>.
- 749 Giorgini, M, U Bernardo, M M Monti, A G Nappo, and M Gebiola. 2010. “Rickettsia Symbionts  
750 Cause Parthenogenetic Reproduction in the Parasitoid Wasp *Pnigalio Soemius*  
751 (Hymenoptera: Eulophidae).” *Applied and Environmental Microbiology* 76 (8): 2589–99.  
752 <https://doi.org/10.1128/AEM.03154-09>.
- 753 Gomila, Margarita, Arantxa Peña, Magdalena Mulet, Jorge Lalucat, and Elena García-Valdés.  
754 2015. “Phylogenomics and Systematics in *Pseudomonas*.” *Frontiers in Microbiology* 6.  
755 <https://www.frontiersin.org/article/10.3389/fmicb.2015.00214>.
- 756 Graham, Linda E, Marie T Trest, Susan Will-Wolf, Naomi S Miicke, Lauren M Atonio, Michael  
757 J Piotrowski, and Jennifer J Knack. 2018. “Microscopic and Metagenomic Analyses of  
758 *Peltigera Ponojensis* (Peltigerales, Ascomycota).” *International Journal of Plant Sciences*  
759 179 (3): 241–55. <https://doi.org/10.1086/696534>.
- 760 Guidetti, R., R. O. Schill, R. Bertolani, T. Dandekar, and M. Wolf. 2009. “New Molecular Data  
761 for Tardigrade Phylogeny, with the Erection of *Paramacrobiotus* Gen. Nov.” *Journal of*  
762 *Zoological Systematics and Evolutionary Research* 47 (4): 315–21.  
763 <https://doi.org/10.1111/J.1439-0469.2009.00526.X>.
- 764 Guidetti, Roberto, Lorena Rebecchi, Roberto Bertolani, Kjell J Ingemar, Reinhardt Møbjerg  
765 Kristensen, and Michele Cesari. 2016. “Morphological and Molecular Analyses on  
766 *Richtersius* (Eutardigrada) Diversity Reveal Its New Systematic Position and Lead to the  
767 Establishment of a New Genus and a New Family within Macrobiotioidea.” *Zoological*  
768 *Journal of the Linnean Society* 178: 834–45. <https://doi.org/10.1111/zoj.12428>.
- 769 Guidetti, Roberto, Matteo Vecchi, Agnese Ferrari, Irene L.G. Newton, Michele Cesari, and  
770 Lorena Rebecchi. 2020. “Further Insights in the Tardigrada Microbiome: Phylogenetic  
771 Position and Prevalence of Infection of Four New Alphaproteobacteria Putative  
772 Endosymbionts.” *Zoological Journal of the Linnean Society* 188 (3): 925–37.  
773 <https://doi.org/10.1093/zoolinnean/zlzl28>.
- 774 Guil, N., R. Guidetti, M. Cesari, T. Marchioro, L. Rebecchi, and A. Machordom. 2022.  
775 “Molecular Phylogenetics, Speciation, and Long Distance Dispersal in Tardigrade  
776 Evolution: A Case Study of the Genus *Milnesium*.” *Molecular Phylogenetics and*  
777 *Evolution*, January, 107401. <https://doi.org/10.1016/J.YMPEV.2022.107401>.

- 778 Hagimori, Tetsuya, Yoshihisa Abe, Shuichi Date, and Kazuki Miura. 2006. “The First Finding of  
779 a Rickettsia Bacterium Associated with Parthenogenesis Induction Among Insects.” *Current*  
780 *Microbiology* 52 (2): 97–101. <https://doi.org/10.1007/s00284-005-0092-0>.
- 781 Hernández-García, Juan Alfredo, Carlos Iván Briones-Roblero, Flor N Rivera-Orduña, and  
782 Gerardo Zúñiga. 2017. “Revealing the Gut Bacteriome of Dendroctonus Bark Beetles  
783 (Curculionidae: Scolytinae): Diversity, Core Members and Co-Evolutionary Patterns.”  
784 *Scientific Reports* 7 (1): 13864. <https://doi.org/10.1038/s41598-017-14031-6>.
- 785 Höfte, Monica, and Paul De Vos. 2006. “Plant Pathogenic Pseudomonas Species.” In *Plant-*  
786 *Associated Bacteria*, edited by Samuel S Gnanamanickam, 507–33. Dordrecht: Springer  
787 Netherlands. [https://doi.org/10.1007/978-1-4020-4538-7\\_14](https://doi.org/10.1007/978-1-4020-4538-7_14).
- 788 Horgan, Finbarr G, Thanga Suja Srinivasan, Eduardo Crisol-Martínez, Maria Liberty P Almazan,  
789 Angelee Fame Ramal, Ricardo Oliva, Ian L Quibod, and Carmencita C Bernal. 2019.  
790 “Microbiome Responses during Virulence Adaptation by a Phloem-Feeding Insect to  
791 Resistant near-Isogenic Rice Lines.” *Ecology and Evolution* 9 (20): 11911–29.  
792 <https://doi.org/https://doi.org/10.1002/ece3.5699>.
- 793 Jiang, Long-Qian, De-Feng An, Kun Zhang, Gui-Ding Li, Xin-Yu Wang, Lei Lang, Ming-Guo  
794 Jiang, Li-Song Wang, Cheng-Lin Jiang, and Yi Jiang. 2020. “Nakamurella Albus Sp. Nov.:  
795 A Novel Actinobacterium Isolated from a Lichen Sample.” *Current Microbiology* 77 (8):  
796 1896–1901. <https://doi.org/10.1007/s00284-020-01928-1>.
- 797 Johnke, Julia, Philipp Dirksen, and Hinrich Schulenburg. 2020. “Community Assembly of the  
798 Native *C. Elegans* Microbiome Is Influenced by Time, Substrate and Individual Bacterial  
799 Taxa.” *Environmental Microbiology* 22 (4): 1265–79.  
800 <https://doi.org/https://doi.org/10.1111/1462-2920.14932>.
- 801 Jönsson, K. Ingemar, Elke Rabbow, Ralph O. Schill, Mats Harms-Ringdahl, and Petra Rettberg.  
802 2008. “Tardigrades Survive Exposure to Space in Low Earth Orbit.” *Current Biology* 18  
803 (17): R729–31. <https://doi.org/10.1016/j.cub.2008.06.048>.
- 804 Kaczmarek, Łukasz, Milena Roszkowska, Izabela Poprawa, Kamil Janelt, Hanna Kmita,  
805 Magdalena Gawlak, Edyta Fiałkowska, and Monika Mioduchowska. 2020. “Integrative  
806 Description of Bisexual Paramacrobrotus Experimentalis Sp. Nov. (Macrobrotidae) from  
807 Republic of Madagascar (Africa) with Microbiome Analysis.” *Molecular Phylogenetics and*  
808 *Evolution* 145 (December 2019). <https://doi.org/10.1016/j.ympev.2019.106730>.
- 809 Kajtoch, Łukasz, and Nela Kotásková. 2018. “Current State of Knowledge on Wolbachia  
810 Infection among Coleoptera: A Systematic Review.” *PeerJ* 6: e4471–e4471.
- 811 Karamura, G., Julian Smith, David Studholme, Jerome Kubiriba, and E. Karamura. 2007.  
812 “Comparative Pathogenicity Studies of the Xanthomonas Vasicola Species on Maize,  
813 Sugarcane and Banana.” *African Journal of Plant Science* 9 (9): 385–400.
- 814 Karstens, Lisa, Mark Asquith, Sean Davin, Damien Fair, W Thomas Gregory, Alan J Wolfe,  
815 Jonathan Braun, and Shannon McWeeney. 2019. “Controlling for Contaminants in Low-  
816 Biomass 16S rRNA Gene Sequencing Experiments.” *MSystems* 4 (4): e00290-19.  
817 <https://doi.org/10.1128/mSystems.00290-19>.
- 818 Kido, Kazutaka, Rie Adachi, Masaru Hasegawa, Kazutaka Yano, Yasufumi Hikichi, Shigeharu  
819 Takeuchi, Tae Atsuchi, and Yuichi Takikawa. 2008. “Internal Fruit Rot of Netted Melon  
820 Caused by Pantoea Ananatis (=Erwinia Ananas) in Japan.” *Journal of General Plant*  
821 *Pathology* 74 (4): 302–12. <https://doi.org/10.1007/s10327-008-0107-3>.
- 822 Kim, Soo-Jin, Hayoung Cho, Jae-Ho Joa, Moriyuki Hamada, Jae-Hyung Ahn, Hang-Yeon  
823 Weon, and Soon-Wo Kwon. 2017. “Nakamurella Intestinalis Sp. Nov., Isolated from the

- 824 Faeces of *Pseudorhynchus Japonicus*.” *International Journal of Systematic and*  
825 *Evolutionary Microbiology* 67 (8): 2970–74. <https://doi.org/10.1099/ijsem.0.002059>.
- 826 Kinchin, Ian M. 1994. *The Biology of Tardigrades*. London: Portland Press Ltd.
- 827 Kinchin, Ian M. 1996. “Morphometric Analysis of *Ramazzottius Varieornatus* (Hypsibiidae:  
828 Eutardigrada).” *Zoological Journal of the Linnean Society* 116: 51–60.  
829 <https://academic.oup.com/zoolinnean/article/116/1-2/51/2684283>.
- 830 Kraaijeveld, Ken, Barbara M Reumer, Laurence Mouton, Natacha Kremer, Fabrice Vavre, and  
831 Jacques J M van Alphen. 2011. “Does a Parthenogenesis-Inducing *Wolbachia* Induce  
832 Vestigial Cytoplasmic Incompatibility?” *Die Naturwissenschaften* 98 (3): 175–80.  
833 <https://doi.org/10.1007/s00114-010-0756-x>.
- 834 Krantz, Stefanie L., Thomas G. Benoit, and Clark W. Beasley. 1999. “Phytopathogenic Bacteria  
835 Associated with Tardigrada.” *Zoologischer Anzeiger* 238: 259–60.
- 836 Lawson, Eileen T, Timothy A Mousseau, Rebecca Klaper, Mark D Hunter, and John H Werren.  
837 2001. “*Rickettsia* Associated with Male-Killing in a Buprestid Beetle.” *Heredity* 86 (4):  
838 497–505. <https://doi.org/10.1046/j.1365-2540.2001.00848.x>.
- 839 Li, Jiale, Chengcheng Li, Ming Wang, Lixiang Wang, Xiaobo Liu, Chenglong Gao, Lili Ren,  
840 and Youqing Luo. 2021. “Gut Structure and Microbial Communities in *Sirex Noctilio*  
841 (Hymenoptera: Siricidae) and Their Predicted Contribution to Larval Nutrition.” *Frontiers*  
842 *in Microbiology* 12. <https://www.frontiersin.org/article/10.3389/fmicb.2021.641141>.
- 843 Li, Xiaoying, Lu Fu, Changlong Chen, Wangwang Sun, Yu Tian, and Hua Xie. 2020.  
844 “Characteristics and Rapid Diagnosis of *Pectobacterium Carotovorum* Ssp. Associated With  
845 Bacterial Soft Rot of Vegetables in China.” *Plant Disease* 104 (4): 1158–66.  
846 <https://doi.org/10.1094/PDIS-05-19-1033-RE>.
- 847 Ma, Bing, Michael E Hibbing, Hye-Sook Kim, Ralph M Reedy, Iris Yedidia, Jane Breuer,  
848 Jeffrey Breuer, et al. 2007. “Host Range and Molecular Phylogenies of the Soft Rot  
849 Enterobacterial Genera *Pectobacterium* and *Dickeya*.” *Phytopathology*® 97 (9): 1150–63.  
850 <https://doi.org/10.1094/PHTO-97-9-1150>.
- 851 Martin, Bryan D, Daniela Witten, and Amy D Willis. 2020. “Modeling Microbial Abundances  
852 and Dysbiosis with Beta-Binomial Regression.” *Ann Appl Stat* 14 (1): 94–115.  
853 <https://doi.org/10.1214/19-aos1283>.
- 854 Martinović, Tijana, Iñaki Odriozola, Tereza Mašínová, Barbara Doreen Bahmann, Petr Kohout,  
855 Petr Sedlák, Kristina Merunková, et al. 2021. “Temporal Turnover of the Soil Microbiome  
856 Composition Is Guild-Specific.” *Ecology Letters* 24 (12): 2726–38.  
857 <https://doi.org/https://doi.org/10.1111/ele.13896>.
- 858 McMurdie, Paul J., and Susan Holmes. 2013. “Phyloseq: An R Package for Reproducible  
859 Interactive Analysis and Graphics of Microbiome Census Data.” *PLoS ONE* 8 (4): e61217.  
860 <https://doi.org/10.1371/journal.pone.0061217>.
- 861 Mew, T. W. 1993. “Focus on Bacterial Blight of Rice.” *Plant Disease* 77: 5–12.  
862 <https://doi.org/10.1094/pd-77-0005>.
- 863 Meyer, Harry A. 2006. “Small-Scale Spatial Distribution Variability in Terrestrial Tardigrade  
864 Populations.” In *Hydrobiologia*. <https://doi.org/10.1007/s10750-005-1412-x>.
- 865 ———. 2013. “Terrestrial and Freshwater Tardigrada of the Americas.” *Zootaxa* 3747 (1): 001–  
866 071. <https://doi.org/10.11646/zootaxa.3747.1.1>.
- 867 Meyer, Harry A. 2008. “Distribution of Tardigrades in Florida.” *Southeastern Naturalist* 7 (1):  
868 91–100. <https://doi.org/10.1656/S407.sl>.
- 869 Michalczyk, Łukasz, Weronika Weńnicz, Marcus Frohme, Łukasz Kaczmarek, Weronika

- 870 Welnicz, Marcus Frohme, and Łukasz Kaczmarek. 2012. “Redescriptions of Three  
871 Milnesium Doyère, 1840 Taxa (Tardigrada: Eutardigrada: Milnesiidae), Including the  
872 Nominal Species for the Genus.” *Zootaxa* 3154 (3154): 1–20.  
873 <https://doi.org/10.11646/zootaxa.3154.1.1>.
- 874 Miller, William R. 1997. “Tardigrades: Bears of the Moss.” *Kansas School Naturalist* 43 (3): 1–  
875 15.
- 876 Mioduchowska, Monika, Bartosz Nitkiewicz, Milena Roszkowska, Uroš Kačarević, Piotr  
877 Madanecki, Tom Pinceel, Tadeusz Namiotko, Bartłomiej Goldyn, and Łukasz Kaczmarek.  
878 2021. “Taxonomic Classification of the Bacterial Endosymbiont Wolbachia Based on Next-  
879 Generation Sequencing: Is There Molecular Evidence for Its Presence in Tardigrades?”  
880 *Genome*, May. <https://doi.org/10.1139/gen-2020-0036>.
- 881 Mogle, Matthew J., Scott A. Kimball, William R. Miller, and Richard D. McKown. 2018.  
882 “Evidence of Avian-Mediated Long Distance Dispersal in American Tardigrades.” *PeerJ*  
883 2018 (7). <https://doi.org/10.7717/peerj.5035>.
- 884 Moro, Matheus Sartori, Xing Wu, Wei Wei, Lucas William Mendes, Kerry Clint Allen, José  
885 Baldin Pinheiro, Steven J Clough, and Maria Imaculada Zucchi. 2021. “Characterization  
886 and Comparison of Intestinal Bacterial Microbiomes of Euschistus Heros and Piezodorus  
887 Guildinii Collected in Brazil and the United States.” *Frontiers in Microbiology* 12.  
888 <https://www.frontiersin.org/article/10.3389/fmicb.2021.769965>.
- 889 Nelson, Diane R., Paul J. Bartels, and Stephen R. Fegley. 2020. “Environmental Correlates of  
890 Tardigrade Community Structure in Mosses and Lichens in the Great Smoky Mountains  
891 National Park (Tennessee and North Carolina, USA).” *Zoological Journal of the Linnean  
892 Society* 188 (3): 913–24. <https://doi.org/10.1093/ZOOLINNEAN/ZLZ043>.
- 893 Nelson, Diane R., and Karen L McGlothlin. 1996. “A New Species of Calohypsibius (Phylum  
894 Tardigrada, Eutardigrada) from Roan Mountain, Tennessee-North Carolina, U.S.A.”  
895 *Zoological Journal of the Linnean Society* 116: 167–74.  
896 <https://academic.oup.com/zoolinnea/article/116/1-2/167/2684365>.
- 897 Ng, James C. K., and Bryce W. Falk. 2006. “Virus-Vector Interactions Mediating Nonpersistent  
898 and Semipersistent Transmission of Plant Viruses.” *Annual Review of Phytopathology* 44  
899 (1): 183–212. <https://doi.org/10.1146/annurev.phyto.44.070505.143325>.
- 900 O’Leary, Nuala A, Mathew W Wright, J Rodney Brister, Stacy Ciufu, Diana Haddad, Rich  
901 McVeigh, Bhanu Rajput, et al. 2016. “Reference Sequence (RefSeq) Database at NCBI:  
902 Current Status, Taxonomic Expansion, and Functional Annotation.” *Nucleic Acids Research*  
903 44 (D1): D733–45. <https://doi.org/10.1093/nar/gkv1189>.
- 904 Ordax, Mónica, Jaime E Piquer-Salcedo, Ricardo D Santander, Beatriz Sabater-Muñoz, Elena G  
905 Biosca, María M López, and Ester Marco-Noales. 2015. “Medfly *Ceratitis Capitata* as  
906 Potential Vector for Fire Blight Pathogen *Erwinia Amylovora*: Survival and Transmission.”  
907 *PLOS ONE* 10 (5): e0127560. <https://doi.org/10.1371/journal.pone.0127560>.
- 908 Palmer, Marike, Emma T Steenkamp, Martin P A Coetzee, Wai-Yin Chan, Elritha van Zyl,  
909 Pieter De Maayer, Teresa A Coutinho, et al. 2017. “Phylogenomic Resolution of the  
910 Bacterial Genus *Pantoea* and Its Relationship with *Erwinia* and *Tatumella*.” *Antonie van  
911 Leeuwenhoek* 110 (10): 1287–1309. <https://doi.org/10.1007/s10482-017-0852-4>.
- 912 Passera, Alessandro, Stéphane Compant, Paola Casati, Maria Giovanna Maturo, Giovanna  
913 Battelli, Fabio Quaglino, Livio Antonielli, et al. 2019. “Not Just a Pathogen? Description of  
914 a Plant-Beneficial *Pseudomonas Syringae* Strain.” *Frontiers in Microbiology* 10.  
915 <https://www.frontiersin.org/article/10.3389/fmicb.2019.01409>.

- 916 Paudel, Sujan, Shefali Dobhal, Anne M Alvarez, and Mohammad Arif. 2020. “Taxonomy and  
917 Phylogenetic Research on *Ralstonia Solanacearum* Species Complex: A Complex Pathogen  
918 with Extraordinary Economic Consequences.” *Pathogens (Basel, Switzerland)* 9 (11): 886.  
919 <https://doi.org/10.3390/pathogens9110886>.
- 920 Remenant, Benoît, Jean-Charles de Cambiaire, Gilles Cellier, Jonathan M Jacobs, Sophie  
921 Mangenot, Valérie Barbe, Aurélie Lajus, et al. 2011. “*Ralstonia Syzygii*, the Blood Disease  
922 Bacterium and Some Asian *R. Solanacearum* Strains Form a Single Genomic Species  
923 despite Divergent Lifestyles.” *PLoS One* 6 (9): e24356–e24356.  
924 <https://doi.org/10.1371/journal.pone.0024356>.
- 925 Saati-Santamaría, Zaki, Ezequiel Peral-Aranega, Encarna Velázquez, Raúl Rivas, and Paula  
926 García-Fraile. 2021. “Phylogenomic Analyses of the Genus *Pseudomonas* Lead to the  
927 Rearrangement of Several Species and the Definition of New Genera.” *Biology* 10 (8): 782.  
928 <https://doi.org/10.3390/biology10080782>.
- 929 Sasu, M A, I Seidl-Adams, K Wall, J A Winsor, and A G Stephenson. 2010. “Floral  
930 Transmission of *Erwinia Tracheiphila* by Cucumber Beetles in a Wild *Cucurbita Pepo*.”  
931 *Environmental Entomology* 39 (1): 140–48. <https://doi.org/10.1603/EN09190>.
- 932 Schram, Mark D, and Paul G Davison. 2012. “Irwin Loops—A History and Method of  
933 Constructing Homemade Loops.” *Transactions of the Kansas Academy of Science* 115 (1–  
934 2): 35–40. <https://doi.org/10.1660/062.115.0106>.
- 935 Schuster, Rolf, and Hartmut Greven. 2007. “A Long-Term Study of Population Dynamics of  
936 Tardigrades in the Moss *Rhytidiadelphus Squarrosus* (Hedw.) Warnst.” *Journal of*  
937 *Limnology* 66 (Suppl. 1): 141–51.
- 938 ———. 2013. “Reproductive Traits of *Macrobiotus Hufelandi* during a Long-Term Field Study  
939 with Notes on *Paramacrobiotus Richtersi* and *Diphyscon Pingue* (Eutardigrada).” *Journal of*  
940 *Limnology* 72 (s1): 166–74. <https://doi.org/10.4081/jlimnol.2013.s1.e21>.
- 941 Shapiro, Lori R, Erin D Scully, Timothy J Straub, Jihye Park, Andrew G Stephenson, Gwyn A  
942 Beattie, Mark L Gleason, et al. 2016. “Horizontal Gene Acquisitions, Mobile Element  
943 Proliferation, and Genome Decay in the Host-Restricted Plant Pathogen *Erwinia*  
944 *Tracheiphila*.” *Genome Biology and Evolution* 8 (3): 649–64.  
945 <https://doi.org/10.1093/gbe/evw016>.
- 946 Silby, Mark W, Craig Winstanley, Scott A C Godfrey, Stuart B Levy, and Robert W Jackson.  
947 2011. “*Pseudomonas* Genomes: Diverse and Adaptable.” *FEMS Microbiology Reviews* 35  
948 (4): 652–80. <https://doi.org/10.1111/j.1574-6976.2011.00269.x>.
- 949 Stavrínides, John, Jodi K McCloskey, and Howard Ochman. 2009. “Pea Aphid as Both Host and  
950 Vector for the Phytopathogenic Bacterium *Pseudomonas Syringae*.” *Applied and*  
951 *Environmental Microbiology* 75 (7): 2230–35. <https://doi.org/10.1128/AEM.02860-08>.
- 952 Tibbs-Cortes, Laura E., Bienvenido W. Tibbs-Cortes, and William R. Miller. 2020. “Tardigrades  
953 of Hardin County, Iowa: Seven New Records from Iowa, USA.” *Transactions of the Kansas*  
954 *Academy of Science* 123 (1–2): 203–12. <https://doi.org/10.1660/062.123.0117>.
- 955 Tuo, Li, Fei-Na Li, Zhen Pan, Inchio Lou, Min Guo, Simon Ming-Yuen Lee, Li Chen, Lin Hu,  
956 and Cheng-Hang Sun. 2016. “*Nakamurella Endophytica* Sp. Nov., a Novel Endophytic  
957 Actinobacterium Isolated from the Bark of *Kandelia Candel*.” *International Journal of*  
958 *Systematic and Evolutionary Microbiology* 66 (3): 1577–82.  
959 <https://doi.org/10.1099/ijsem.0.000923>.
- 960 Vecchi, Matteo, Irene L.G. G Newton, Michele Cesari, Lorena Rebecchi, and Roberto Guidetti.  
961 2018. “The Microbial Community of Tardigrades: Environmental Influence and Species

- 962 Specificity of Microbiome Structure and Composition.” *Microbial Ecology* 76 (2): 467–81.  
963 <https://doi.org/10.1007/s00248-017-1134-4>.
- 964 Walterson, Alyssa M, and John Stavriniades. 2015. “Pantoea: Insights into a Highly Versatile and  
965 Diverse Genus within the Enterobacteriaceae.” *FEMS Microbiology Reviews* 39 (6): 968–  
966 84. <https://doi.org/10.1093/femsre/fuv027>.
- 967 Wang, Qiong, George M Garrity, James M Tiedje, and James R Cole. 2007. “Naive Bayesian  
968 Classifier for Rapid Assignment of RRNA Sequences into the New Bacterial Taxonomy.”  
969 *Applied and Environmental Microbiology* 73 (16): 5261–67.  
970 <https://doi.org/10.1128/AEM.00062-07>.
- 971 Werren, John H, Laura Baldo, and Michael E Clark. 2008. “Wolbachia: Master Manipulators of  
972 Invertebrate Biology.” *Nature Reviews Microbiology* 6 (10): 741–51.  
973 <https://doi.org/10.1038/nrmicro1969>.
- 974 Willis, Amy D, and Bryan D Martin. 2020. “Estimating Diversity in Networked Ecological  
975 Communities.” *Biostatistics*, May. <https://doi.org/10.1093/biostatistics/kxaa015>.
- 976 Xin, Xiu-Fang, Brian Kvitko, and Sheng Yang He. 2018. “Pseudomonas Syringae: What It  
977 Takes to Be a Pathogen.” *Nature Reviews Microbiology* 16 (5): 316–28.  
978 <https://doi.org/10.1038/nrmicro.2018.17>.
- 979 Xue, Hui, Xiangzhen Zhu, Li Wang, Kaixin Zhang, Dongyang Li, Jichao Ji, Lin Niu, et al. 2021.  
980 “Gut Bacterial Diversity in Different Life Cycle Stages of Adelphocoris Suturalis  
981 (Hemiptera: Miridae).” *Frontiers in Microbiology* 12.  
982 <https://www.frontiersin.org/article/10.3389/fmicb.2021.670383>.
- 983 Yan, Xiao-Rui, Ming-Sheng Chen, Chao Yang, Ming-Biao An, Hong-Ying Li, Hui-Chang Shi,  
984 and Li Tuo. 2020. “Nakamurella Flava Sp. Nov., a Novel Endophytic Actinobacterium  
985 Isolated from Mentha Haplocalyx Briq.” *International Journal of Systematic and  
986 Evolutionary Microbiology* 70 (2): 835–40. <https://doi.org/10.1099/ijsem.0.003831>.
- 987 Young, Alexander, and Ken Clifton. 2015. “Tardigrades Inhabit Lichen and Moss in Smith Rock  
988 State Park, Oregon.” *Bulletin of the California Lichen Society* 22 (2): 48–53.
- 989 Yuliar, Yanetri Asi Nion, and Koki Toyota. 2015. “Recent Trends in Control Methods for  
990 Bacterial Wilt Diseases Caused by Ralstonia Solanacearum.” *Microbes and Environments*  
991 30 (1): 1–11. <https://doi.org/10.1264/jsme2.ME14144>.
- 992 Zawierucha, Krzysztof, Artur Trzebny, Jakub Buda, Elizabeth Bagshaw, Andrea Franzetti,  
993 Mirosława Dabertid, and Roberto Ambrosini. 2022. “Trophic and Symbiotic Links between  
994 Obligate-Glacier Water Bears (Tardigrada) and Cryoconite Microorganisms.” Edited by  
995 Brenda A. Wilson. *PLOS ONE* 17 (1): e0262039.  
996 <https://doi.org/10.1371/JOURNAL.PONE.0262039>.
- 997 Zhang, Fan, Maureen Berg, Katja Dierking, Marie-Anne Félix, Michael Shapira, Buck S Samuel,  
998 and Hinrich Schulenburg. 2017. “Caenorhabditis Elegans as a Model for Microbiome  
999 Research.” *Frontiers in Microbiology* 8.  
1000 <https://www.frontiersin.org/article/10.3389/fmicb.2017.00485>.
- 1001 Zhang, Yucheng, and Sai Qiu. 2015. “Examining Phylogenetic Relationships of Erwinia and  
1002 Pantoea Species Using Whole Genome Sequence Data.” *Antonie van Leeuwenhoek* 108 (5):  
1003 1037–46. <https://doi.org/10.1007/s10482-015-0556-6>.
- 1004 Zhou, Guo-Wei, Fei Zheng, Xiao-Ting Fan, Ming-Jun Li, Qing-Ye Sun, Yong-Guan Zhu, and  
1005 Xiao-Ru Yang. 2022. “Host Age Increased Conjugal Plasmid Transfer in Gut Microbiota of  
1006 the Soil Invertebrate Caenorhabditis Elegans.” *Journal of Hazardous Materials* 424:  
1007 127525. <https://doi.org/https://doi.org/10.1016/j.jhazmat.2021.127525>.



1008 Zimmermann, Johannes, Nancy Obeng, Wentao Yang, Barbara Pees, Carola Petersen, Silvio  
1009 Waschina, Kohar A Kissoyan, et al. 2020. “The Functional Repertoire Contained within the  
1010 Native Microbiota of the Model Nematode *Caenorhabditis Elegans*.” *The ISME Journal* 14  
1011 (1): 26–38. <https://doi.org/10.1038/s41396-019-0504-y>.

## 1012 **Figure Legends**

1013  
1014 **Figure 1.** Principal Coordinates Analyses conducted by contrast based on Bray-Curtis distance.  
1015 Location names (Location 1 through Location 6) are abbreviated as L1 through L6. **(A)**  
1016 Tardigrade samples from different locations overlap one another, as do **(B)** tardigrades isolated  
1017 from lichen and moss. However, **(C)** tardigrade and substrate samples are clearly separated, and  
1018 **(D)** 2019 and 2020 tardigrade samples are mostly separated.

1019 **Figure 2.** Relative abundance of top 10 identifiable phyla shown across all contrasts. Location  
1020 names (Location 1 through Location 6) are abbreviated as L1 through L6.

1021  
1022 **Figure 3.** Relative abundance of top 10 genera (identifiable at least to family level) shown across  
1023 all contrasts. Location names (Location 1 through Location 6) are abbreviated as L1 through L6.

1024  
1025 **Figure S1.** Collection locations map. The map of Iowa, USA shows the sampled counties  
1026 outlined in red. The inset shows collection sites within Hardin and Franklin counties identified  
1027 by location number.

1028  
1029 **Figure S2.** Relative abundance of top 10 identifiable phyla shown across all samples. Location  
1030 names (Location 1 through Location 6) are abbreviated as L1 through L6.

1031  
1032 **Figure S3.** Relative abundance of top 10 genera (identifiable at least to family level) shown  
1033 across all samples. Location names (Location 1 through Location 6) are abbreviated as L1  
1034 through L6.

1035  
1036 **Figure S4.** Principal Coordinates Analysis of all samples based on Bray-Curtis distance.  
1037 Location names (Location 1 through Location 6) are abbreviated as L1 through L6. Substrate  
1038 samples are clearly separated from the tardigrade samples along Axis 1. On Axis 2, samples  
1039 from 2020 are generally clustered away from 2019 samples. Samples from different locations  
1040 and from lichen and moss overlap.

1041

## 1042 **Tables**

1043 **Table 1.** Details of samples used in the experiment. Samples are arranged by contrast; when  
1044 samples were included in multiple contrasts, these samples appear more than once in the table.  
1045 “Loc. code” is the location code for a given orchard (e.g., L1 is Location 1) and “# trees”

1046 indicates the number of trees sampled at that location for a given contrast. From each sample of  
1047 moss or lichen, three to six replicates were extracted, identified in the “Replicate codes” column.  
1048

1049

Contrast	Year	Loc. code	GPS location	Sample details	# trees	Replicate codes
<b>Contrast 1:</b> Tardigrades from same substrate (lichen) in different locations.	2019	L1	42.56N, -93.49W	Tardigrades from lichen	3	L1_19_Tr1_li1-3, L1_19_Tr2_li1-3, L1_19_Tr3_li1-3
	2019	L2	42.43N, -93.07W	Tardigrades from lichen	4	L2_19_Tr1_li1-3, L2_19_Tr2_li1-3, L2_19_Tr3_li1-3, L2_19_Tr4_li1-3
	2019	L3	42.44N, -93.11W	Tardigrades from lichen	3	L2_19_Tr1_li1-3, L2_19_Tr2_li1-3, L2_19_Tr3_li1-3
	2019	L4	42.42N, -93.08W	Tardigrades from lichen	4	L4_19_Tr1_li1-3, L4_19_Tr2_li1-3, L4_19_Tr3_li1-3, L4_19_Tr4_li1-3
	2019	L5	42.40N, -93.31W	Tardigrades from lichen	4	L5_19_Tr1_li1-3, L5_19_Tr2_li1-3, L5_19_Tr3_li1-3, L5_19_Tr4_li1-3
	2019	L6	42.69N, -93.22W	Tardigrades from lichen	5	L6_19_Tr1_li1-3, L6_19_Tr2_li1-3, L6_19_Tr3_li1-3, L6_19_Tr4_li1-3, L6_19_Tr5_li1-3
<b>Contrast 2:</b> Tardigrades from different substrates (moss vs. lichen) on the same tree	2019	L2	42.43N, -93.07W	Tardigrades from lichen	3	L2_19_Tr1_li1-3, L2_19_Tr2_li1-3, L2_19_Tr3_li1-3
	2019	L2	42.43N, -93.07W	Tardigrades from moss	3	L2_19_Tr1_mo1-3, L2_19_Tr2_mo1-3, L2_19_Tr3_mo1-3
<b>Contrast 3:</b> Tardigrades vs. their substrate (lichen).	2020	L1	42.56N, -93.49W	Tardigrades from lichen	4	L1_20_Tr1_li1-5, L1_20_Tr2_li1-5, L1_20_Tr2_li1-5, L1_20_Tr4_li1-5
	2020	L1	42.56N, -93.49W	Lichen only	4	L1_20_Tr1_sub1-6, L1_20_Tr2_sub1-5, L1_20_Tr2_sub1-5, L1_20_Tr4_sub1-4
<b>Contrast 4:</b> Tardigrades from the same trees in different years.	2019	L1	42.56N, -93.49W	Tardigrades from lichen	3	L1_19_Tr1_li1-3, L1_19_Tr2_li1-3, L1_19_Tr3_li1-3
	2020	L1	42.56N, -93.49W	Tardigrades from lichen	3	L1_20_Tr1_li1-5, L1_20_Tr2_li1-5, L1_20_Tr2_li1-5

1050 **Table 2.** Alpha diversity measures Shannon and Simpson estimated for each level of each  
 1051 contrast of interest. Within each contrast and diversity measure, estimates that are significantly  
 1052 different from one another (Benjamini-Hochberg corrected  $P$  value <0.05) share no letters.

Contrast	Level of Contrast	Shannon	Simpson
1: Location	Location 1	1.570 <sup>a</sup>	0.437 <sup>a</sup>
	Location 2	3.545 <sup>b</sup>	0.244 <sup>b</sup>
	Location 3	0.970 <sup>c</sup>	0.779 <sup>c</sup>
	Location 4	2.093 <sup>d</sup>	0.466 <sup>a</sup>
	Location 5	1.605 <sup>a</sup>	0.683 <sup>d</sup>
	Location 6	3.830 <sup>b</sup>	0.255 <sup>b</sup>
2: Moss vs. Lichen	Moss	2.929 <sup>a</sup>	0.175 <sup>a</sup>
	Lichen	2.926 <sup>a</sup>	0.205 <sup>a</sup>
3: Tardigrades vs. Substrate	Tardigrade	2.895 <sup>a</sup>	0.279 <sup>a</sup>
	Substrate	6.673 <sup>b</sup>	0.004 <sup>b</sup>
4: Year	2019	1.448 <sup>a</sup>	0.447 <sup>a</sup>
	2020	1.566 <sup>a</sup>	0.365 <sup>b</sup>

1053

#### 1054 Supplemental Table Legends

1055 **Table S1.** Mothur classification and decontam score for all OTUs with more than 10 reads in  
 1056 experimental samples. OTUs with decontam score below the 0.25 threshold are marked as  
 1057 contaminants.

1058

1059 **Table S2.** BLAST results for OTUs with more than 10 total reads in experimental samples.  
 1060 BLAST+ v2.11.0 was used to query representative sequences for each OTU against a database  
 1061 generated from the NCBI 16S RefSeq collection. The 15 hits with the lowest E-values are given  
 1062 for each OTU.

1063

1064 **Table S3.** RDP Classifier results for OTUs with more than 10 total reads in experimental  
 1065 samples. Representative sequences for each OTU were uploaded to the RDP Classifier webtool  
 1066 (<https://rdp.cme.msu.edu/classifier/classifier.jsp>), version 2.11 using 16S rRNA training set 18.

1067

1068 **Table S4.** Relative abundance of all analyzed OTUs in each sample.

1069 **Table S5.** Relative abundance of each OTU by level of Contrast 1 (Location).

1070 **Table S6.** Relative abundance of each OTUs in moss and in lichen (*i.e.*, at each level of Contrast  
 1071 2).

1072 **Table S7.** Relative abundance of each OTU in tardigrades and in their lichen substrate (*i.e.*, at  
 1073 each level of Contrast 3).

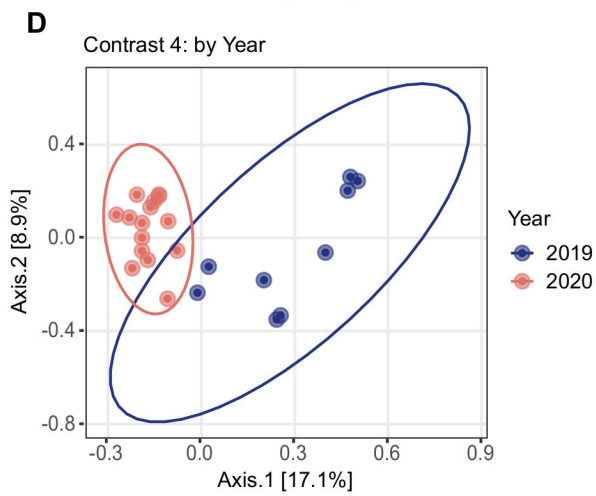
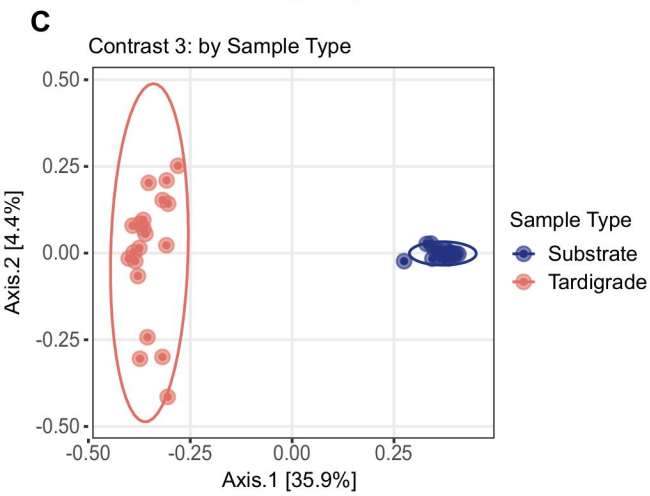
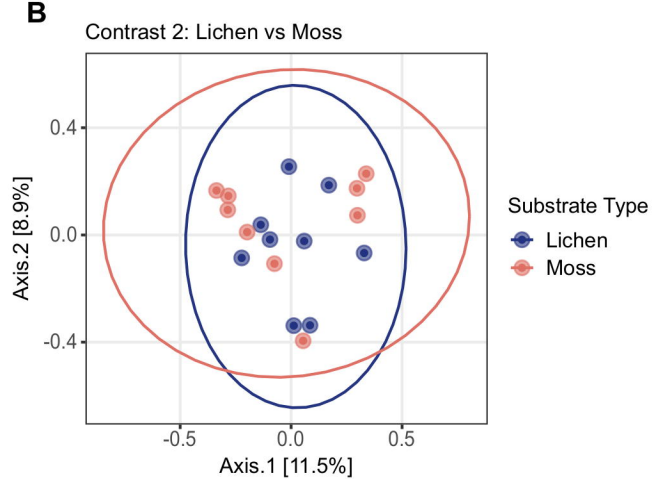
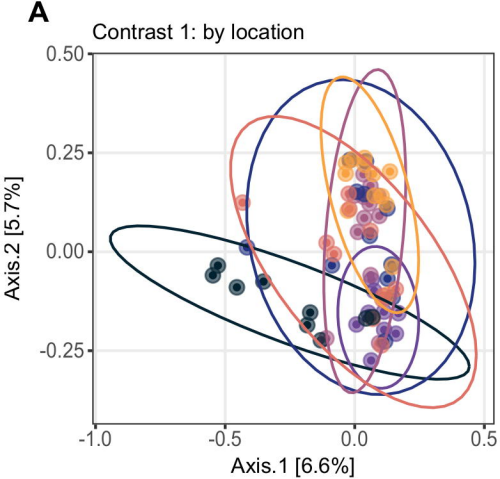
1074 **Table S8.** Relative abundance of each OTU in tardigrades in 2019 and in 2020 (*i.e.*, at each level  
 1075 of Contrast 4).

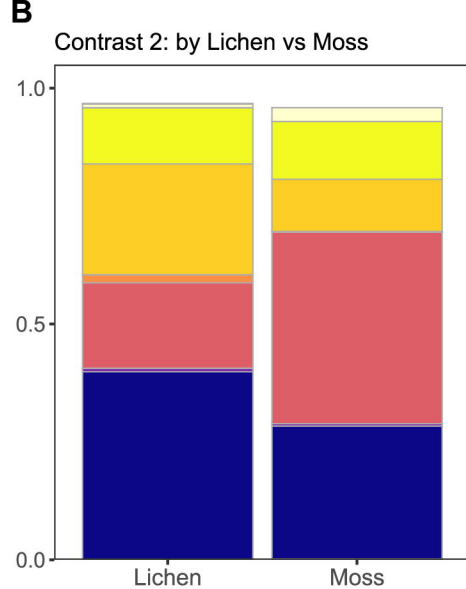
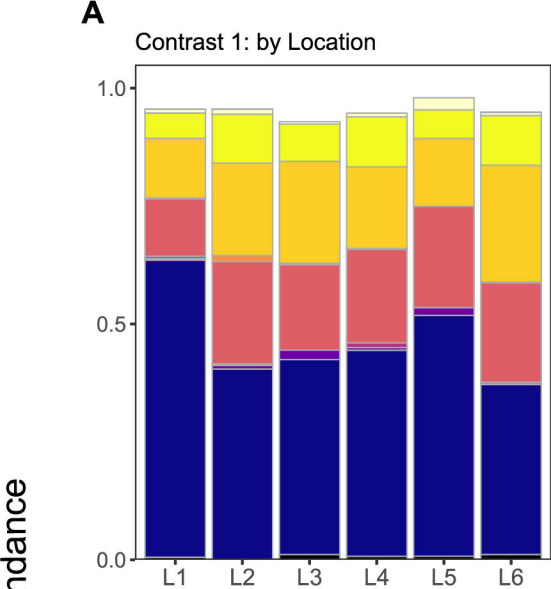
1076 **Table S9.** Differentially abundant and variable taxa across contrasts. Corncob was used to  
1077 identify significantly (Benjamini-Hochberg corrected  $P$  value  $< 0.05$ ) differentially abundant and  
1078 variable taxa across four contrasts of interest. Genus names are presented including their phylum,  
1079 class, order, and family names to prevent ambiguities.

1080 **Table S10.** Relative abundance of top 10 identifiable phyla across levels of each contrast.

1081

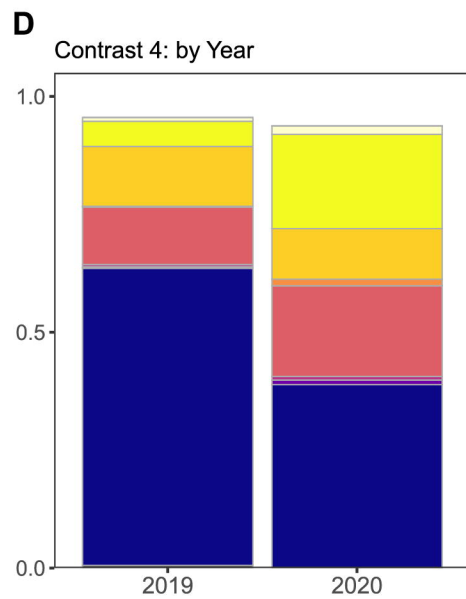
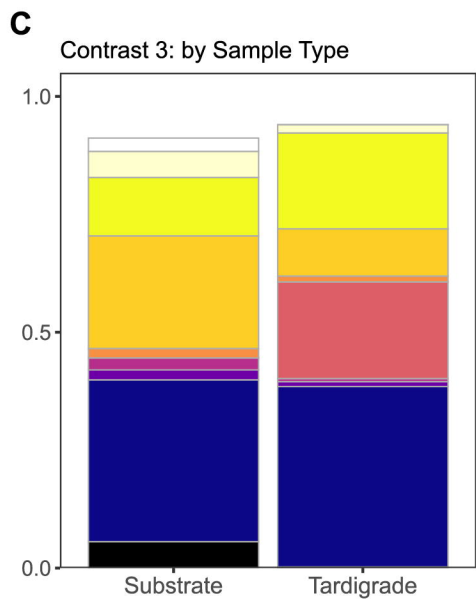
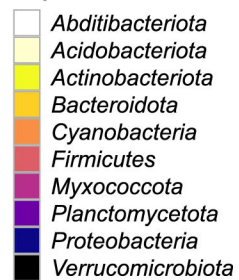
1082 **Table S11.** Relative abundance of top 10 genera (identifiable at least to family level) across  
1083 levels of each contrast.

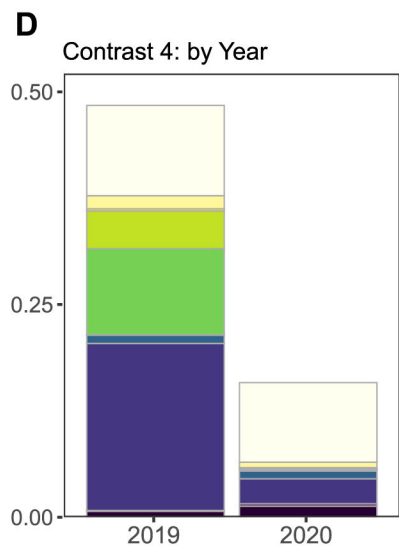
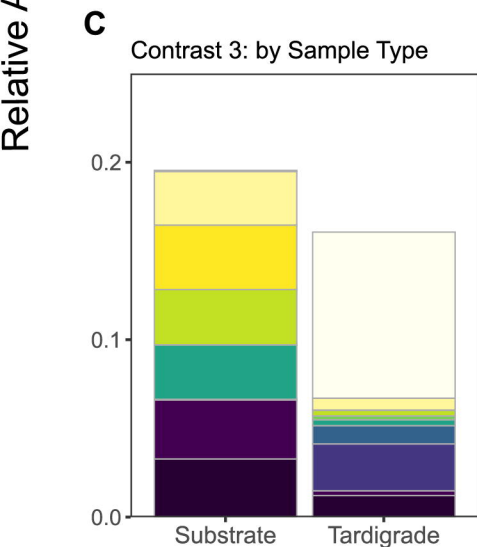
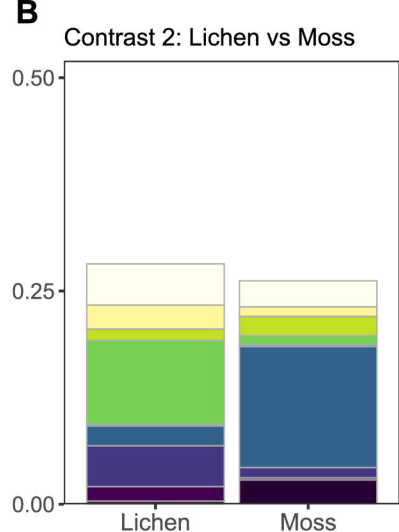
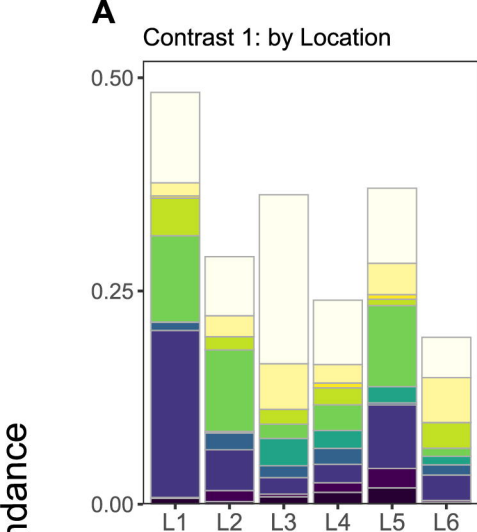




Relative Abundance

Phylum





Genus

- Bradyrhizobium*
- Chitinophagaceae* unclassified
- Chthoniobacter*
- Comamonadaceae* unclassified
- Enterobacteriaceae* unclassified
- Hymenobacter*
- Listeria*
- Pseudomonas*
- Sphingomonadaceae* unclassified
- Sphingomonas*