Competitive outcomes between native and invasive plants are linked to shifts in the bacterial rhizosphere microbiome

Marina L. LaForgia[1], Hannah Kang[2], Cassandra L. Ettinger[3,4*]

[1]Department of Environmental Science and Policy, University of California, Davis, CA, USA

[2]Department of Plant Sciences, University of California, Davis, CA, USA

[3]Genome Center, University of California, Davis, CA, USA

[4]Department of Evolution and Ecology, University of California, Davis, CA, USA

* Corresponding author; email: clettinger@ucdavis.edu

Abstract

Background

Rhizosphere microbiomes have received growing attention in recent years for their role in plant health, stress tolerance, soil nutrition, and invasive species dominance. Still, relatively little is known about how these microbial communities are altered under plant competition, and even less about whether these shifts are tied to competitive outcomes between native and invasive plant species. We investigated the structure and diversity of rhizosphere bacterial and fungal microbiomes of native annual forbs and invasive annual grasses individually and in competition using high-throughput amplicon sequencing of the bacterial 16S rRNA gene and the fungal ITS region. We assessed how significant shifts in key microbial families correlate to plant competitive responses through changes in biomass (log competitive response ratios).

Results

We find that bacterial diversity and structure differ between invasive grasses and native forbs, but fungal diversity and structure do not. Further, bacterial community structures under competition are distinct from both individual forb and grass bacterial community structures. We identified four bacterial families (Burkholderiaceae, Methylophilaceae, Clostridiaceae_1, and Fibrobacteraceae) that varied in relative abundance between treatments and that were significantly correlated with plant competitive responses.

Conclusions

Invasive grass dominance may be partially due to effects on the rhizosphere community of native forbs, with changes in specific bacterial families potentially benefiting grasses at the expense of native forbs. Our study underscores the importance of considering plant-rhizosphere interactions for understanding outcomes of plant invasion on grassland ecosystems.

Keywords: rhizosphere, microbiome, bacteria, fungi, competition, invasive species, plant-microbe interactions, annual grassland

Background

Plants and their associated soil microbial communities have coevolved complex dynamic relationships through time. The soil directly surrounding plant roots, known as the rhizosphere, is home to an abundance and diversity of microbes distinct from the surrounding bulk soil microbial community [1–4]. Microbes in the rhizosphere play vital roles in plant health [1], nutrition [5], and stress tolerance [6,7]. Though rhizosphere microbial composition is driven predominantly by soil particle size, pH, and moisture content [8–10], these communities can also be highly host plant-specific [11,12], with plants exhibiting strong local adaptations to their home microbial community [13–15]. Further, there is growing evidence that microbial community composition varies in the presence of certain neighbors [16], and can even affect competitive outcomes between plants [17–19] with potentially broad consequences for plant community assembly, nutrient cycling, and ecosystem resilience [20–23].

Microbes can affect plant-host competition both directly via resource availability and indirectly through community interactions. For example, microbes can make nitrogen more readily available to plants through the processes of decomposition, nitrification, and nitrogen fixation [23,24], which could preferentially benefit hosts capable of quickly taking up resources [25,26] thereby increasing these plants' competitive success relative to species with slower uptake rates [27]. Plants may also indirectly compete for beneficial plant-growth-promoting microbes, with these microbes boosting root growth and soil exploration of some species more

70 than others [4,28,29]. Conversely, competition with a novel plant, such as an invader, may bring
71 resident plants into contact with novel pathogens [30]. These competitive interactions can lead to
72 the loss of specialized microbes in the inferior competitor [31] and drive the resulting microbial
73 community to resemble that of the dominant competitor [18,19]. Furthermore, microbial
74 community changes can feed back to affect plant competitive ability [19], with the potential to
75 drive either coexistence or exclusion [32].

76 Shifts in the microbiome are a potentially important contributor to the dominance of
77 invasive plants and their threats to native species. Although there is strong evidence for local
78 adaptation between plants and their home microbiome, invasive plants are relatively novel
79 players, causing disruptions in important plant-microbe interactions [33] by increasing soil
80 microbial activity [34], reducing microbial biomass [35], decreasing microbial diversity [36],
81 increasing nitrification rates [25], and changing microbiome composition [37,38]. These plant-
82 soil feedbacks have the potential to further benefit the invader [38,39], selectively harm natives
83 [40], and/or benefit other invaders [41]. While it is known that invaders and natives may harbor
84 distinct microbial communities, and that competition between host plants can affect microbiome
85 composition (and vice versa), little is known of how changes in the abundance of specific
86 microbial taxa may be tied to the competitive dominance of invaders and/or the competitive
87 inferiority of natives.

88 Serpentine soils in California annual grassland communities are characterized by high
89 levels of heavy metals and magnesium and low levels of essential plant nutrients, and as a result,
90 are home to a unique community of native annual forbs adapted to growing in these conditions.
91 While some of these soils are rocky and shallow, other serpentine areas are deeper and more
92 finely textured and therefore hold more water and nitrogen [42], making them more favorable to

93 invasive annual grass establishment and growth. Invasive annual grasses are highly competitive,
94 spread rapidly, and produce an enormous amount of seed each year. Their invasion into finer-
95 textured serpentine areas is promoted through nutrient addition and increased rainfall [42], which
96 leads to a fungal community more like that of heavily-invaded nonserpentine soils [43]. In
97 native-dominated serpentine areas, microbes have been shown to increase seedling survival [44]
98 and facilitate their ability to thrive in heavy metal soils [45]. With the invasion of grasses,
99 changes in the microbiome may strengthen the advantage that these grasses have over native
100 forbs if they are able to recruit beneficial microbes away from natives or harbor novel microbes
101 harmful to native forbs. Alternatively, locally adapted microbes may be harming invasive
102 grasses, helping native forbs to persist in these areas.

103 We sought to understand how competition between native forbs and invasive grasses
104 shifts the bacterial and fungal rhizosphere microbiomes, and how these shifts may be shaping
105 competitive outcomes in this community. Our main questions were:

106 (Q1) Do microbiomes differ between invasive grasses and native forbs?

107 (Q2) Do microbiomes under grass-forb competition differ from the microbiomes of (a)
108 invasive grasses and (b) native forbs?

109 (Q3) If so, are microbiomes under grass-forb competition sourced more from invasive
110 grasses, native forbs, or equally from both?

111 (Q4) Given compositional changes in the microbiome under grass-forb competition,
112 which specific microbial families are driving these changes?

113 (Q5) Are changes in the relative abundance of these microbial families correlated with
114 the competitive responses of forbs and grasses to each other?

To answer these questions, we conducted a manipulative shade-house experiment in which forbs and grasses were grown in field-collected soil both individually and in grass-forb pairs. Individual rhizosphere microbiomes were sampled for each species grown alone, joint rhizosphere microbiomes were sampled for each species pair, and high throughput amplicon sequencing was used to identify both bacterial and fungal rhizosphere communities. To assess how microbiomes differed between invasive grasses and native forbs (Q1), and between each of these groups individually and in competition (Q2), we looked at differences in community structure and composition as well as alpha diversity. We evaluated the contribution of individual grass and forb microbiomes to the joint microbiomes of grass-forb pairs (Q3) by calculating the proportion of the joint microbiome predicted to be sourced from either the forb or the grass microbiome. We then used differential abundance analysis to identify which microbial families were driving any observed changes (Q4). Finally, to understand whether the relative abundances of these microbial families may be tied to the competitive responses of grasses and forbs (Q5), we evaluated the relationship of biomass-based log competitive response ratios of forbs and grasses to each microbial family's relative abundance. To corroborate whether these relationships may be driven by either the forb or the grass, we also calculated the proportion of each family in the joint microbiome predicted to originate from either the forb or the grass microbiome. For each step, we looked at effects in both the bacterial and the fungal rhizosphere. We focus our analysis on group-level results (i.e. native forb, invasive grass, and grass-forb pairs) after ensuring differences in microbiomes were attributable to groups rather than individual plant species. We also accounted for species-level differences with random effects in mixed models.

Methods

138 *Experimental set-up*

139 In spring and summer 2017, we collected soil and seed from an annual grassland at the
140 University of California McLaughlin Natural Reserve [2] in the Inner North Coast Range (N
141 38°52', W 122°26'). The site has a Mediterranean climate with cool, wet winters (mean annual
142 precipitation 2000-2019 of 738 mm [3]) and dry, hot summers. Soil was collected from an area
143 of the reserve with deep, finely-textured serpentine soils and seeds were collected nearby from
144 six common native annual forb species (Fig. 1a; *Lasthenia californica, Clarkia purpurea,*
145 *Agoseris heterophylla, Calycadenia pauciflora, Hemizonia congesta,* and *Plantago erecta)* and
146 three dominant invasive annual grasses (Fig. 1b; *Avena fatua, Taeniatherum caput-medusae,* and
147 *Bromus hordeaceus*). In December 2017, seeds were sown into physan-washed pots with a
148 mixture of 65% field-collected soil and 35% sterile autoclaved sand to improve drainage. Each
149 species was grown alone (five replicates per species) to assess the microbial communities of
150 individual plant species. To assess the effects of competition between natives and invasives, each
151 forb species was grown with each grass species in a pairwise factorial design for a total of 18
152 different species combinations with five replicates per pair. Pots were placed in a shade-house
153 open to natural temperature variation in the UC Davis Orchard Park Greenhouse and soil
154 moisture was maintained with an automatic drip irrigation system (Fig. 1c).

155 *Sample collection*

156 In April 2018, we sampled rhizosphere microbial communities by gently shaking off soil
157 from plant roots, then submerging the roots in autoclaved nanopure water in a 50 mL conical
158 tube and vortexing to obtain the rhizosphere soil similar to Edwards et al. [1]. We sampled
159 rhizospheres prior to flowering to minimize differences in composition due to developmental
160 stage [16,36]. Conical tubes were then centrifuged at 4000 g for 1 minute to obtain rhizosphere

161 soil pellets. In plant mixtures, we sampled the joint rhizosphere microbiome as roots were
162 completely interwoven, suggesting strong interactions between individual rhizospheres.
163 To assess the competitive response of each species, we harvested, dried, and weighed
164 aboveground plant biomass and used it to calculate log response ratios. Roots were not included
165 due to the difficulty of identifying roots to species in the competition treatment. Log response
166 ratio was calculated as ln(weight grown together/mean weight grown alone) [46]. Values below
167 zero indicate competition with more negative values signifying a stronger response to
168 competition.
169 *Molecular methods and sequence generation*
170 We randomized samples using a random number generator prior to DNA extraction. We
171 then extracted the DNA from samples using the MoBio PowerSoil DNA Extraction kit (MO BIO
172 Laboratories, Inc., Carlsbad, CA, USA) with one minor modification to manufacturer's
173 instructions: samples were bead beat on the "homogenize" setting for 1 minute during step 5.
174 Samples were then sent to the Integrated Microbiome Resource (IMR) at Dalhousie University to
175 amplify and sequence the 16S rRNA gene using the 515FB-806RB primer set [47,48] and ITS2
176 region using the ITS86F-ITS4R primer set [49]. The amplification and sequencing process is
177 described in Comeau et al. [50]. In addition to experimental samples, we sent five subsamples of
178 the background field-collected soil-sand mix, three replicates each of a positive technological
179 control using DNA extracted from the ZymoBIOMICS Microbial Community Standard (Zymo
180 Research Inc., Irvine, CA, USA), and a negative technological control where no sample was
181 added during DNA extraction to the IMR for sequencing. The sequence reads generated for this
182 16S rRNA gene and ITS region amplicon project were deposited at Genbank under accession no.
183 PRJNA666893.

184 *Sequence processing*

185 Sequencing primers were removed using the Cutadapt (v. 2.3) program and the resulting
186 sequences were then processed using the DADA2 workflow in R . 16S rRNA gene amplicon
187 sequences were trimmed at 280 base pairs for forward reads and 250 base pairs for reverse reads
188 based on their error profiles to maintain a quality score above 10. ITS region amplicon sequences
189 were not trimmed to preserve the ability to merge reads due to the inherent length variation of
190 the ITS region. Both 16S rRNA gene and ITS region amplicon reads were then truncated at the
191 first quality score of 2 and reads with an expected error greater than 2 were removed. We then
192 merged paired end reads, created an Amplicon Sequence Variant table (ASV), and identified
193 chimeric reads using removeBimeraDenovo, which were subsequently removed prior to
194 downstream analyses (~4.1% of sequence reads for 16S rRNA gene, ~0.93% for ITS region).

195 For 16S rRNA gene ASVs, we assigned taxonomy using the RDP Naive Bayesian
196 Classifier algorithm and the SILVA high quality ribosomal RNA database v. 132 [51]. For ITS
197 ASVs, taxonomy was inferred with the UNITE (v. 8.0) database modified to include a
198 representative ITS amplicon sequence for each host plant, i.e. *H. congesta* (MF964056.1), *C.*
199 *purpurea* (MG235764.1), *P. erecta* (AY101909.1), *A. fatua* (FJ794719.1), *L. californica*
200 (AY043514.1), *A. heterophylla* (AY218965.1), *T. caput-medusae* (AJ608153.1), *B. hordeaceus*
201 (KP987344.1) [52,53]. ITS-x (v. 1.1.1) was then run on the unique ITS ASVs to pull out only
202 sequences that had a fungal ITS region based on kingdom-specific hidden markov models [54].
203 ITS-x was unable to detect a fungal ITS region in 744 of the ASVs (~13.46% of ASVs). These
204 ASVs were thus considered putatively non-fungal and were removed from downstream analysis.

205 Decontam's prevalence method was used to identify putative contaminants with a
206 threshold of 0.5 which will identify sequences that have a higher prevalence in negative controls

207 than in true samples [55]. This method identified 55 contaminants in the 16S rRNA gene data
208 and 32 in the ITS amplicon data which were then removed from the analysis.
209 For the 16S rRNA gene dataset, unique ASVs were aligned using the MAFFT Alignment
210 Program (v 7.402) on XSEDE with standard parameters through the CIPRES Science Gateway
211 V. 3.3 (https://www.phylo.org/) [56,57]. We then built a phylogenetic tree using FastreeMP on
212 XSEDE with standard parameters also through CIPRES [58] and rooted the tree using an
213 archaeal outgroup. We then created a phyloseq object from the resulting 16S ASV table, Silva
214 taxonomy table, phylogeny, and mapping file using the phyloseq package in R [59]. Sequences
215 that were taxonomically assigned to chloroplasts and mitochondria were then removed and
216 samples were rarefied to 9,434 reads per sample for comparison purposes.
217 For the ITS region data, we created a phyloseq object from the ITS ASV table, UNITE
218 taxonomy table, and mapping file using the phyloseq package in R. Samples were rarefied to
219 7,557 reads per sample for comparison purposes.
220 *Data analysis (Q1-Q2)*
221 To evaluate how rhizosphere communities varied in structure (beta diversity) between
222 groups and in competition, we used the vegan package [60] in R to conduct a Principal
223 Coordinates Analysis (PCoA) on weighted UniFrac of each sample for rarefied 16S rRNA gene
224 data and on Bray-Curtis dissimilarities for rarefied ITS region data. We tested for significant
225 differences in mean centroids between groups (native forbs, invasive grasses, grass-forb pairs)
226 using the adonis function to perform a permutational multivariate analysis of variance
227 (PERMANOVA) and between mean dispersions using the betadisper function in vegan. We then
228 conducted post-hoc analyses using the adonis.pair function with Benjamini-Hochberg p-value
229 corrections [61]. We also investigated whether these groups varied compared to background soil

230 (i.e. the community in the soil-sand mix at the start of the experiment) by performing
231 PERMANOVA tests to ascertain whether plant hosts were drivers of community assembly.
232 Finally, we investigated the degree of species-level variation within both grasses and forbs by
233 running PERMANOVA tests on each group separately to evaluate whether there was more
234 variation within or between groups.

235 To assess how rhizosphere communities varied in alpha diversity between grasses, forbs
236 and grass-forb pairs, we calculated Shannon diversity of each sample using the estimate_richness
237 function in the phyloseq package, then conducted a Kruskal-Wallis test to test whether alpha
238 diversity varied between these groups. We then conducted post-hoc analyses using a Dunn Test
239 with Benjamini-Hochberg p-value corrections.

240 *Data analysis (Q3)*

241 To evaluate whether a larger proportion of the joint microbiome community was
242 predicted to be sourced from forbs or grasses, we used SourceTracker, a Bayesian source
243 tracking classifier [62]. First we trained SourceTracker on the rarified ASV tables of forbs,
244 grasses, and background soil and then tested the trained Baysian model on the grass-forb pair
245 joint microbiomes to estimate the proportion of ASVs originating from each source. The trained
246 Baysian model assumes that joint microbiomes contain a combination of colonists from known
247 and unknown sources (i.e. anything not sampled here such as air, water, etc.) and estimates the
248 fraction of ASVs detected in the joint microbiome that originated from grasses, forbs,
249 background soil or unknown habitats. We used Wilcoxon signed-rank tests to assess significant
250 differences in the SourceTracker predicted proportions between different sources for the
251 bacterial community and fungal community, and then also tested for differences in the
252 proportions of each individual source between the two communities.

253 *Data Analysis (Q4)*

254 We used differential abundance analysis to identify microbial families whose relative
255 abundance varied between groups in order to investigate which families were driving observed
256 differences in the community structure and composition. To do this, we first summed the raw
257 read counts of each full data set (i.e. not rarefied) into families using the tax_glom function in
258 phyloseq. We used the DESeq2 R package [63] on the family counts to examine the $\log_2$fold
259 change (i.e. differential abundance) of families between pairwise contrasts of groups (forbs,
260 grasses, and grass-forb pairs). We focused on families that were identified as differentially
261 abundant in at least one pairwise comparison between forbs, grasses and grass-forb pairs with
262 Bonferroni corrected p-values < 0.01.

263 *Data analysis (Q5)*

264 To understand whether the relative abundances of these microbial families may be tied to
265 the competitive responses of grasses and forbs, we first identified which functional group was
266 competitively dominant by testing for differences in log competitive response ratios between
267 grasses and forbs in a linear mixed effect model with functional group as our predictor and a
268 random intercept for plant species combination to account for species-level differences.

269 To determine if the relative abundance of a specific microbial family was related to forb
270 or grass competitive response, we ran linear mixed effects models for each host group (grass and
271 forb) and each differentially abundant microbial family with log competitive response ratios as
272 the response variable and regularized log abundance of the focal family (calculated using the rld
273 function in the DESeq2 package; hereafter referred to as relative abundance) as the predictor
274 variable. Plant species combination was also included as a random intercept to account for
275 species-level differences.

Finally, we also calculated the proportion of each differentially abundant family in the joint microbiome predicted to originate from either the individual forb or grass microbiomes by grouping ASVs into taxonomic families and then summarizing the fraction of ASVs within each family attributed to each possible source using SourceTracker.

Results

Q1: Do microbiomes differ between invasive grasses and native forbs?

Overall, the rhizosphere bacterial microbiome varied between invasive grasses and native forbs, but the fungal microbiome did not. The structure of forb bacterial microbiomes was significantly different from the structure of grass bacterial microbiomes ($p < 0.001$; Fig. 2). Further, bacterial Shannon diversity (Fig. 3) was significantly higher in forb microbiomes than in grass microbiomes ($p = 0.035$). Conversely, there were no significant differences in the structure of fungal communities among treatments ($p = 0.072$; Fig. S1). There were also no differences in fungal Shannon diversity among treatments ($p = 0.854$; Fig. S2). Further, microbial community structure was significantly different between background soil and all treatment groups for both the bacterial ($p < 0.001$, Fig. S3a) and fungal communities ($p < 0.001$, Fig. S3b).

The structure of individual microbiomes did not vary between forb species (bacterial: $p = 0.069$, Fig. S4a; fungal: $p = 0.755$, Fig. S5a), but did vary between grass species (bacterial $p = 0.013$, Fig. S4b; fungal: $p = 0.589$, Fig. S5b). The bacterial microbiomes of *T. caput-medusae* were distinct from those of *B. hordeaceus* ($p = 0.049$) and *A. fatua* ($p = 0.047$). Statistical differences between grass and forb bacterial microbiomes remained even after removing this species and rerunning the analysis, suggesting larger variation between grass and forb functional groups than within grass or forb groups. We therefore proceeded with the analysis of all species, but focused the remainder of our results at the functional group level.

Q2: Do microbiomes under grass-forb competition differ from the microbiomes of (a) invasive grasses and (b) native forbs?

Grass-forb pairs displayed significantly different joint bacterial microbiomes from microbiomes of both functional groups grown alone (forb: $p < 0.001$; grass: $p < 0.001$; Fig. 2). Grass-forb pair microbiomes also had marginally higher Shannon diversity than grass microbiomes ($p = 0.059$), but did not significantly differ from forb microbiomes ($p = 0.292$). As reported above, there were no significant differences found between treatments in the fungal microbiome (either in structure or diversity), so we did not conduct post-hoc analyses to assess pairwise contrasts between treatment groups.

Q3: Are microbiomes under grass-forb competition sourced more from invasive grasses, native forbs, or equally from both?

For bacteria, similar fractions of the community in competition were predicted to have originated from grasses and from forbs ($p = 0.341$, grass: 40.34 ± 1.33%, forb: 42.13 ± 1.14%). Only relatively low proportions of the bacterial community came from background soil or unknown sources (background: 8.57 ± 0.48%, unknown: 8.96 ± 0.60%). For fungi, the fraction of the community predicted to originate from grasses was marginally higher than that from forbs ($p = 0.066$, grass: 38.88 ± 2.26%, forb: 32.65 ± 2.01%). Additionally, the fraction of the fungal community originating from forbs was significantly lower than the fraction of the bacterial community originating from forbs ($p < 0.001$). A small proportion of the fungal community was predicted to derive from background soil (5.16 ± 0.67%), while a much larger proportion was estimated to be from unknown sources (23.31 ± 2.11%). The contribution of unknown sources to the fungal community was significantly higher than the contribution of unknown sources to the bacterial community ($p < 0.001$).

322 *Q4: Given compositional changes in the microbiome under grass-forb competition, which*
323 *specific microbial families are driving these changes?*
324 Differential abundance analysis revealed a total of 19 bacterial families (Fig. S6a) that
325 varied between treatments, with 16 bacterial families varying between grass-forb pairs and either
326 grass or forb microbiomes and three additional families varying only between grass and forb
327 microbiomes. Of the 16 bacterial families that were differentially abundant under competition,
328 six families varied between both microbiomes of forbs and grasses grown alone and between
329 grass-forb pair microbiomes and individual microbiomes: Methylophilaceae, Fibrobacteraceae,
330 Clostridiaceae_1, Burkholderiaceae, Rhodocyclaceae, and Veillonellaceae (Fig. 4).
331 Methylophilaceae was the only family with higher relative abundance in forbs and
332 subsequently lower relative abundance in grass-forb pairs. Both Fibrobacteraceae and
333 Clostridiaceae_1 were higher in relative abundance in grass than forb microbiomes and were
334 subsequently higher in grass-forb pairs than in forbs alone. The relative abundance of
335 Burkholderiaceae was higher in grasses than in forbs, but decreased in grass-forb pairs.
336 Veillonellaceae displayed higher relative abundances in grasses than in forbs, and remained
337 higher in grass-forb pairs. Finally, the relative abundance of Rhodocyclaceae was higher in
338 grasses than in forbs, and lower in grass-forb pairs than in grasses. Of the remaining 13 families,
339 three families only displayed differential abundances between grasses and forbs and 10 families
340 displayed differential abundances between grass-forb pairs and forbs but not between grasses and
341 forbs (Fig. S7).
342 A total of three fungal families were differentially abundant between treatments (Fig.
343 S6b, Fig. S8). Of these, only Tubeufiaceae showed variation between both individual
344 microbiomes and grass-forb pairs, with higher relative abundances in forbs than grasses and

345 subsequently lower relative abundances in grass-forb pairs than in forbs. Ceratobasidiaceae
346 displayed lower relative abundances in grass-forb pairs than in grasses, and an unclassified
347 family from the order Sebacinales displayed higher abundance in grass-forb pairs than in forbs.
348 *Q5: Are changes in the relative abundance of these microbial families correlated with the*
349 *competitive responses of forbs and grasses to each other?*
350 Grasses were generally dominant over forbs, and this dominance was correlated with the
351 relative abundance of some bacterial families. Native forbs displayed stronger negative log
352 response ratios than invasive grasses ($p = 0.047$; Fig. 5), indicating that forbs were more
353 negatively affected by competition from grasses than the grasses were from forbs. Competitive
354 outcomes did, however, vary depending on the identity of the grass and the identity of the forb
355 within the species pair combination (Fig. S9), indicating a wide range of responses.
356 Of the six families that differentially varied between grass-forb pairs and either grasses or
357 forbs, the relative abundances of Methylophilaceae, Fibrobacteraceae, Clostridiaceae_1, and
358 Burkholderiaceae also displayed correlations with either forb or grass log response ratios (Fig.
359 6). Moreover, those families that displayed negative correlations with forb responses and/or
360 positive correlations with grass responses had higher predicted proportions originating from
361 grass microbiomes, while the one family that displayed the opposite correlations (positively
362 correlated with forb response/negative with grass response) had a higher predicted proportion
363 originating from forb microbiomes (Fig. 7).
364 The relative abundance of Methylophilaceae was significantly positively correlated with
365 log response ratio of forbs (est = 0.48, se = 0.14 , $p < 0.001$), and significantly negatively
366 correlated with log response ratio of grasses (est = -0.32, se = 0.13, $p = 0.021$), indicating that
367 as the relative abundance of this family decreased, forbs responded more negatively to grass

368 competition. Conversely, grasses were negatively correlated with higher relative abundances of
369 this family. This family also showed a higher proportion of ASVs originating from the forb
370 microbiome in grass-forb pairs.

371 The relative abundance of Fibrobacteraceae was significantly negatively correlated with
372 forb log response ratio (est = -0.51, se = 0.19, $p = 0.009$), but was not correlated with grass
373 response (est = -0.07, se = 0.18, $p = 0.719$; Fig 5b), indicating that as the relative abundance of
374 this family increased, the competitive effect of grasses on forbs intensified, while grasses were
375 unaffected by changes in this family. The relative abundance of Clostridiaceae_1 was
376 significantly positively correlated with grass log response ratio (est = 0.35, se = 0.16, $p = 0.036$),
377 but not with forbs (est = -0.26, se = 0.18, $p = 0.139$), indicating that as this family increased, the
378 competitive response of grasses to forbs weakened. A higher predicted proportion of ASVs
379 within these families originated from grasses, suggesting that grasses may have been driving
380 high relative abundance of Fibrobacteraceae and Clostridiaceae_1 in grass-forb pairs.

381 The relative abundance of Burkholderiaceae was negatively correlated with forb log
382 response ratio (est = -0.36 , se = 0.11, $p = 0.002$) and positively correlated with grass log
383 response ratio (est = 0.37, se = 0.11, $p < 0.001$), indicating that as the relative abundance of this
384 family increased, forbs were more negatively affected by competition from grasses and grasses
385 were less negatively affected by competition from forbs. Grasses also contributed a
386 disproportionate number of ASVs from this family to the joint microbiome.

387 In the remaining 15 bacterial families that displayed differential abundances among
388 treatments, we found no significant relationships to log competitive response ratios (Fig. S10).
389 For predicted proportions of these families originating from grasses and forbs, see Fig. S11.

390 Tubeufiaceae, the only fungal family that displayed differential abundance between forbs
391 and grasses, and between grass-forb pairs and forbs, displayed no correlation with either forb or
392 grass log competitive response ratios (Fig. S12). Tubeufiaceae was composed of a higher
393 predicted proportion of ASVs originating from forbs relative to grasses (Fig. S12).
394 Ceratobasidiaceae, which was significantly lower in grass-forb pairs than in grasses but did not
395 vary between grasses and forbs, showed positive correlations of its relative abundance to log
396 response ratios of grasses (est = 0.23, se = 0.04, $p < 0.001$) and negative correlations with forb
397 log response ratios (est = -0.11, se = 0.05, $p = 0.032$), indicating that as the relative abundance of
398 this family increased, forbs were more negatively affected by grasses and grasses were less
399 negatively affected by forbs. We found no other relationships between the remaining
400 differentially abundant fungal family and group log response ratios (Fig. S13).

401 **Discussion**

402 The rhizosphere bacterial community of native forbs is structurally distinct from those of
403 invasive grasses and has higher bacterial alpha diversity. Competition between grasses and forbs
404 in our system correlates with shifts in the bacterial rhizosphere of native forbs towards
405 communities dissimilar to both individual forb and grass microbiomes, with marginally higher
406 diversity than grasses alone. Studies in similar annual grasslands have found little change in
407 bacterial structure with increased invasive grass abundance [43,64,65] but invader dominance
408 has been linked to decreased bacterial diversity in other systems [36,66]. Contrary to Hortal et al.
409 [18] and Lozano et al. [19], we did not find that the new community resembled that of the
410 dominant competitor; instead, during competition, we observed shifts towards a novel
411 assemblage of bacteria distinct from both individual forb and grass microbiomes, and sourced
412 nearly equally from these groups. Competition is therefore associated with shifts in the

413 microbiomes of both invasive grasses and native forbs; however, these changes are linked with
414 more negative consequences for native forbs than invasive grasses.

415 Native annual forbs displayed a stronger negative response to competition than invasive
416 grasses. Plants were well-watered and harvested before shading could become problematic,
417 therefore virtually all competitive effects in this experiment likely resulted mainly from below-
418 ground competition. Given the large role played by microbes in nutrient availability and uptake,
419 observed competitive imbalances, and the substantial differences between forb and grass
420 microbiomes, it is likely these competitive outcomes are mediated by microbes. Invaded
421 grassland soils tend to be depleted in plant available nitrogen [35,64,65], but with increased
422 microbial activity and faster nitrogen cycling [25,34,65]. These microbe-driven changes may
423 selectively benefit fast-growing grasses over natives, especially in nutrient-poor serpentine soils
424 where added nitrogen has been found to preferentially aid invasives [67,68]. Invasive grasses
425 may further benefit from decreased microbial diversity, which has been linked to both decreased
426 nitrogen uptake by forbs and increased nitrogen uptake by grasses [69]. Beyond competition for
427 nutrients, grasses and forbs may also be indirectly competing through recruitment of microbes
428 harmful to natives [70] and/or helpful to invasives [39]. Other studies in serpentine grasslands
429 have shown that soil primed with invasive grass negatively affected the growth of native forbs
430 due to changes in the microbial community [71,72]. While our study was not able to disentangle
431 these various mechanisms, our results provide evidence that compositional shifts in the
432 rhizosphere microbiome may be reinforcing the competitive dominance of these grasses.

433 The relative abundance of 19 bacterial families showed significant variation between
434 grasses, forbs, and grass-forb pairs. Of these 19 families, we found links between bacterial
435 relative abundance and plant competitive response for four: two families were linked to

436 decreased competitive abilities in forbs (Burkholderiaceae, Fibrobacteraceae), one family was
437 linked to increased competitive ability in grasses (Clostridiaceae_1), and one family was linked
438 to both decreased competitive ability of grasses and increased competitive ability of forbs
439 (Methylophilaceae). All four of these families were differentially abundant between forb and
440 grass rhizospheres and between grass-forb pairs and either grasses or forbs.

441 We found only one bacterial family, Methylophilaceae, that was linked to increased
442 competitive ability in forbs. This family was more abundant in forbs than in grasses, and
443 declined in relative abundance in grass-forb pairs. Still, higher relative abundance of
444 Methylophilaceae was associated with a less negative competitive response in forbs and a more
445 negative response in grasses. Bacteria in this family have been found in soils with high heavy
446 metal content and include microbes critical for heavy metal attenuation and immobilization
447 [73,74]. Members of Methylophilaceae also form tight symbioses with plants [75–77] and are
448 even known to promote seed germination and plant growth [76]. This family may include
449 important taxa for serpentine-adapted species, with a decrease in relative abundance driving
450 negative effects through reduced heavy metal attenuation and plant growth. Forbs also
451 contributed a higher percentage of ASVs from this family to the joint grass-forb rhizosphere
452 microbiome, further supporting close associations between native forbs and this group. Invasive
453 grass dominance may therefore be partially due to a decrease in locally adapted microbes similar
454 to Cavalieri et al. [31].

455 Relative abundances of the bacterial families Fibrobacteraceae and Clostridiaceae_1 were
456 higher in grasses than in forbs and remained higher in competition treatments. Grasses were
457 likely driving these higher relative abundances, as they contributed a higher percentage of ASVs
458 from these families to the joint rhizosphere microbiome in pairs. The relative abundance of

459 Fibrobacteraceae was significantly negatively correlated with forb competitive response, but not
460 grass competitive response. Conversely, the relative abundance of Clostridiaceae_1 was
461 positively correlated with grass response, but did not display a relationship with forb response.
462 Although Fibrobacteraceae is typically lower in abundance in soils with high heavy metal
463 content [74], this family is also positively associated with invasive grass dominance [78].
464 Further, both Fibrobacteraceae and Clostridiaceae_1 are known for their cellulose-degrading
465 properties and are often found in rumen [79,80], and therefore may be important taxa for the
466 breakdown of organic matter [81]. These two families also contain known nitrogen-fixers [82–
467 84]. Together, these taxa may be increasing nutrient availability and thereby disproportionately
468 helping fast-growing invasive annual grasses [67,68].

469 The relative abundance of the bacterial family Burkholderiaceae was higher in grasses
470 than in forbs, but was found to be lower in competition. Even with this decrease, grasses may
471 have benefited from their presence - grass competitive response was positively correlated with
472 their relative abundance while forb's displayed a negative competitive response correlation.
473 Grasses also contributed a higher proportion of ASVs from this family to the joint rhizosphere
474 under competition. This family is considered a keystone member in grasslands and is often
475 associated with plants as endophytes or pathogens [85–88]. Burkholderiaceae contains members
476 known for their antimicrobial properties [89–91], nitrogen-fixing abilities [82,92], and
477 competitive dominance, especially in N-limited environments [93] like serpentine. Some
478 members of Burkholderiaceae are even known to suppress fungal pathogens including the turf
479 grass pathogen Rhizoctonia (family Ceratobasidiaceae) [94,95]. It is possible that the
480 Burkholderiacae observed in competition here are either providing positive functional benefits to
481 grasses, or are less harmful to grasses than forbs as pathogens.

482 In contrast to our bacterial rhizosphere findings, we found no significant differences in
483 fungal communities across treatments, either in terms of structure or diversity. This suggests
484 only loose fungal associations with plant hosts and a lack of plant-driven selection for specific
485 fungal taxa, possibly due to functional redundancy of fungi across large geographic scales [96].
486 Within annual grasslands, however, there is evidence of invader-driven fungal shifts [43] and
487 increases in fungal relative abundance [97]. While we found one fungal family that was
488 differentially abundant between both plants grown alone and together (Tubeufiaceae), the only
489 relationship between fungal family relative abundance and competition we found was in
490 Ceratobasidiaceae, which did not vary between grass and forb microbiomes, but was lower in
491 pairs relative to grasses. Ceratobasidiaceae play important ecological roles as plant pathogens,
492 saprotrophs or plant endophytes [98–100]. The negative correlations with forb response and
493 positive correlation with grass response observed here may indicate that this group is acting as a
494 beneficial endophyte of grasses, or may be explained by evolved pathogen resistance in grasses
495 and lack thereof in forbs [101,102].
496 Our limited fungal findings may indeed indicate that fungi are generally less important
497 than bacteria for competitive outcomes between plants [103]. Alternatively, fungi may be
498 important, but complex fungal-fungal interactions at the community level may cancel out fitness
499 effects on plants by beneficial and detrimental fungi [104]. In addition, experimental constraints
500 such as unaccounted fungal regional source pools, plant age at sampling, and methodology may
501 have prevented the observation of fungal relative abundance effects on host plant biomass.
502 Critical fungi are often locally sourced from airborne spores or local watersheds during plant
503 development [96] and thus may have been missing from the shade-house where our experiment
504 took place. This is highlighted in our findings that ~23% of the fungal community was predicted

505 to come from ‘unknown’ sources. Another limitation is plant age at time of sampling, as fungi
506 may form tighter associations later on in plant development or have greater effects on plant
507 fitness over longer time periods [16]. Finally, tightly associated fungi may be root endophytes
508 (e.g. Emam et al. [105]) or found in the rhizoplane (e.g. Edwards et al. [1]), rather than the
509 rhizosphere sampled here.

510 **Conclusions**

511 Our study found that native annual forbs are host to a diverse and distinct community of
512 microbes possibly helping them cope with serpentine’s high heavy metal content, but these close
513 associations are disrupted by invasive grasses. The joint bacterial rhizosphere of invasive grasses
514 and native forbs differed from those of native forbs and invasive grasses grown alone, suggesting
515 that rhizosphere shifts may be related to competition. Both forbs and grasses experienced shifts
516 in their microbiome under competition, but invasive grasses behaved as might be expected of
517 generalists and were notably less affected by these changes. Moreover, the relative abundances
518 of four bacterial families were correlated with plant host competitive outcomes, providing
519 support for the role of microbe-mediated grass dominance in this system. More research is
520 needed to clarify whether the observed changes in plant competitive response were driven by
521 these candidate bacterial families and future work should focus on these families through
522 individual inoculations to tease apart their role in plant growth and competition. While microbial
523 diversity was unaffected by competition in this simplified pairwise experiment, the rhizosphere
524 may become homogenized as grasses invade over larger landscapes [43,66], with selective
525 benefits for grasses over forbs [69]. Increasing native forb abundance in this ecosystem and
526 inoculating soil with locally adapted microbes through restoration efforts may help to alleviate
527 decreases in critical bacteria and lead to better fitness outcomes for native forbs. More generally,

528 our study provides further affirmation of the importance of plant-microbe relationships to the
529 maintenance of ecosystems facing dominance by invasive species.
530
531 **Declarations**
532 *Ethics approval and consent to participate*
533 Not applicable.
534
535 *Consent for publication*
536 Not applicable.
537
538 *Availability of data and material*
539 The datasets and scripts supporting the conclusions of this article are available in the GitHub
540 repository, DOI:10.5281/zenodo.4422181. The sequence reads generated for this 16S rRNA gene
541 and ITS region amplicon project were deposited at Genbank under accession no. PRJNA666893.
542
543 *Competing interests*
544 The authors declare that the research was conducted in the absence of any commercial or
545 financial relationships that could be construed as a potential conflict of interest.
546
547 *Funding*
548 This work was largely supported by grants from the UC Davis Center for Population Biology to
549 MLL and CLE and the UC Davis Center for Population Biology Hardman Foundation Research
550 Award to MLL. This work was also supported in part by a grant awarded to undergraduate

assistant HK from the California Native Plant Society. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

MLL, CLE, and HK conceived of, planned, and carried out the experiment. MLL and CLE processed and analyzed the data and wrote the paper. All authors contributed to the final version of the manuscript.

Acknowledgements

We would like to thank undergraduate intern, Vyvy Ha, with their help on this project, including processing plant biomass and assisting with shade-house related activities. We are also grateful to Paul Aigner for permission to use their photos. Finally, we would like to thank Susan P. Harrison, Andrew M. Latimer and Jonathan A. Eisen for useful conversations and suggestions related to this work, providing lab resources and space and contributing funds to this project.

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Figure 1. (a) Native annual forb species (from left to right, top to bottom): *Clarkia purpurea, Calycadenia pauciflora, Lasthenia californica, Agoseris heterophylla, Plantago erecta,* and *Hemizonia congesta.* (b) Invasive annual grasses (from left to right): *Bromus hordeaceus, Taeniatherum caput-medusae,* and *Avena fatua.* (c) Shade-house set-up. Photos of *C. purpurea*, *P. erecta, H. congesta*, *C. pauciflora*, *B. hordeaceus*, *T. caput-medusae*, and *A. fatua* by Paul Aigner. Remaining photos by authors.

888 **Figure 2.** Bacterial microbiome structure differs between grasses and forbs, and shifts during
889 competition. Principal coordinates analysis (PCoA) visualization of weighted UniFrac distances
890 of bacterial communities associated with the rhizosphere. Points in the ordination are colored and
891 represented by shapes based on the rhizosphere of forbs grown alone (purple circles), grasses
892 grown alone (green triangles) and grass-forb pairs, i.e. competition (yellow squares).

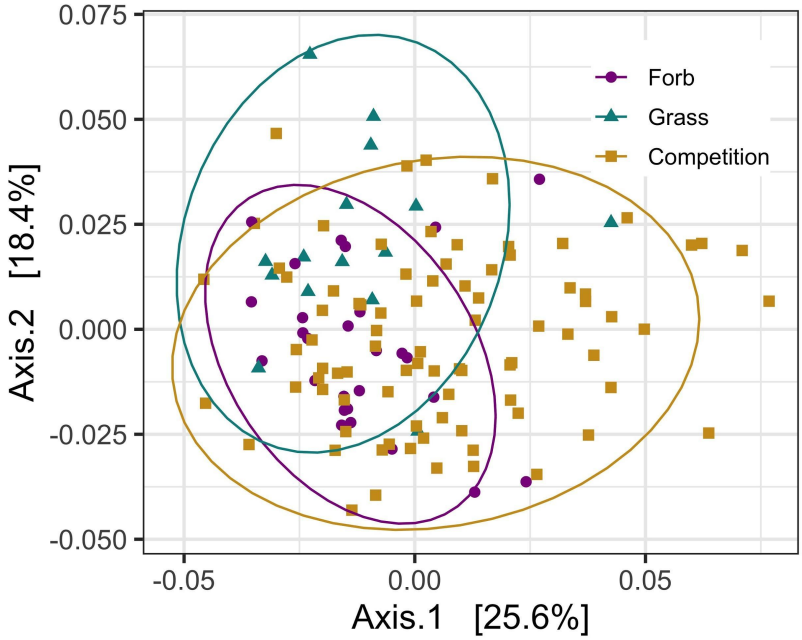

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Figure 3. Shannon diversity of bacterial microbiomes is highest in forbs. Shannon diversity was used to assess alpha diversity for bacterial microbiomes for each treatment (forbs grown alone, grasses grown alone, competition). Comparisons that are significantly different from each other are notated by different letters (e.g. a vs. b).

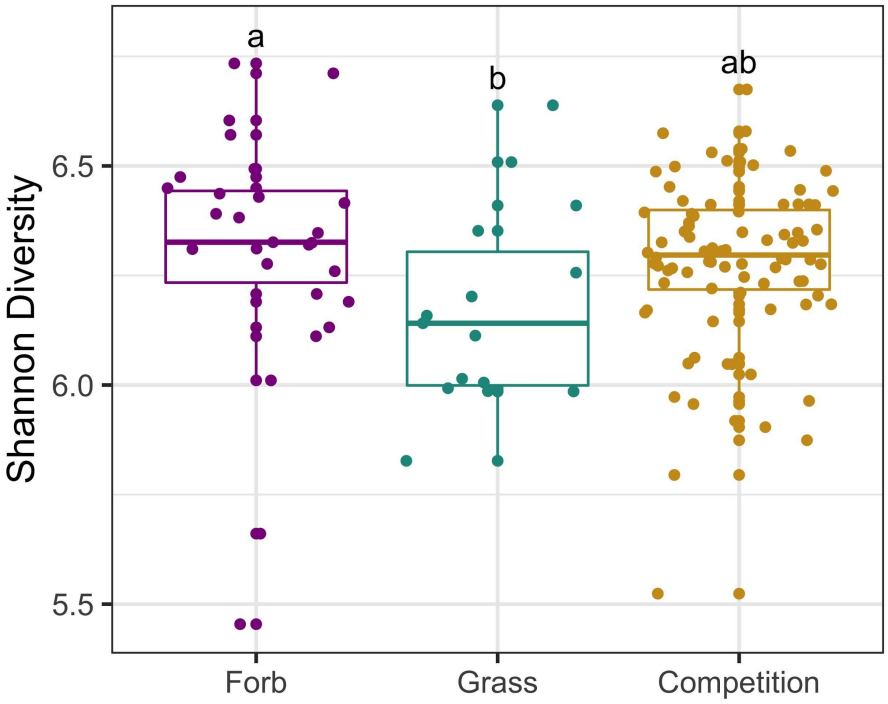

Figure 4. Bacterial families that are differentially abundant between grass and forb microbiomes, and between grass-forb pairs and either grass or forb microbiomes. Using DESeq2, bacterial families were identified whose relative abundance differed significantly between treatments (forbs grown alone, grasses grown alone, grown together in competition). Each plot shows the relative abundance of families for each treatment and comparisons that are significant from each other are notated by different letters (e.g. a vs. b).

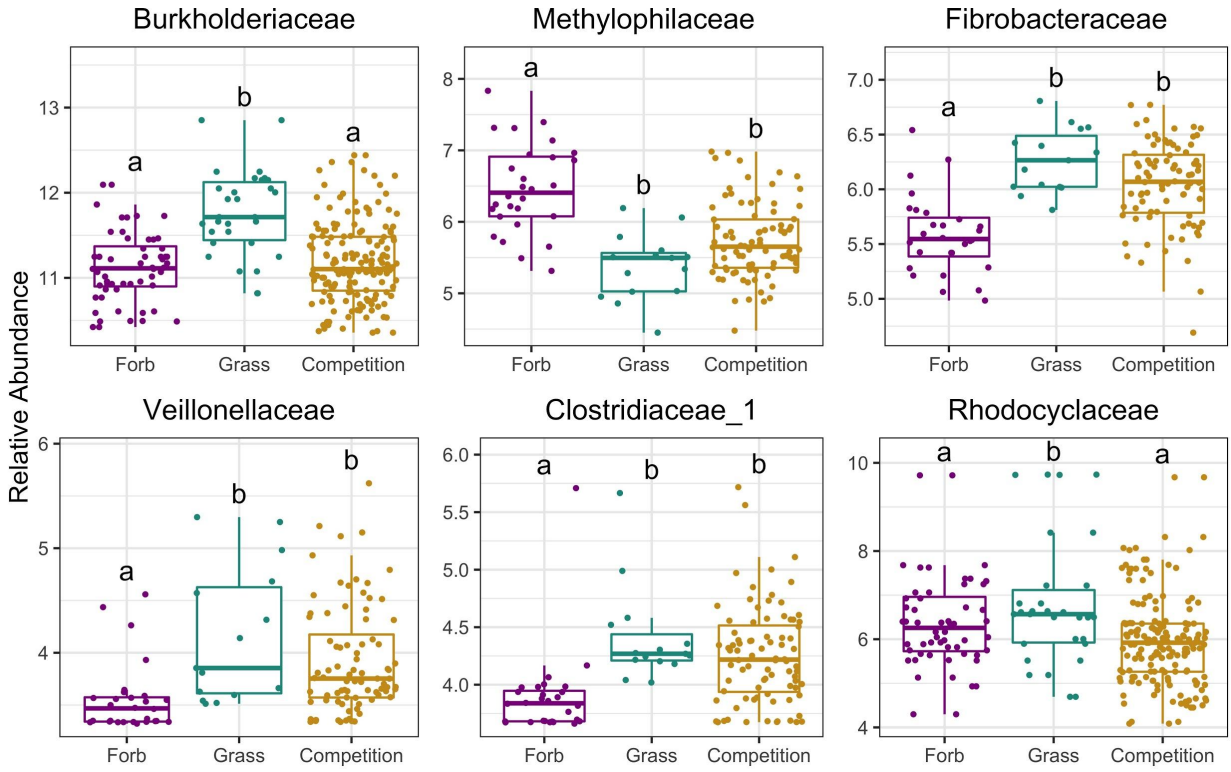

Figure 5. Native forbs were more negatively affected by competition. Log response ratios for forbs and grasses were calculated to determine the dominant competitor and were based on dried aboveground biomass weight. Values below zero indicate competition with more negative values signifying a stronger response to competition. Error bars here represent the standard error of the mean.

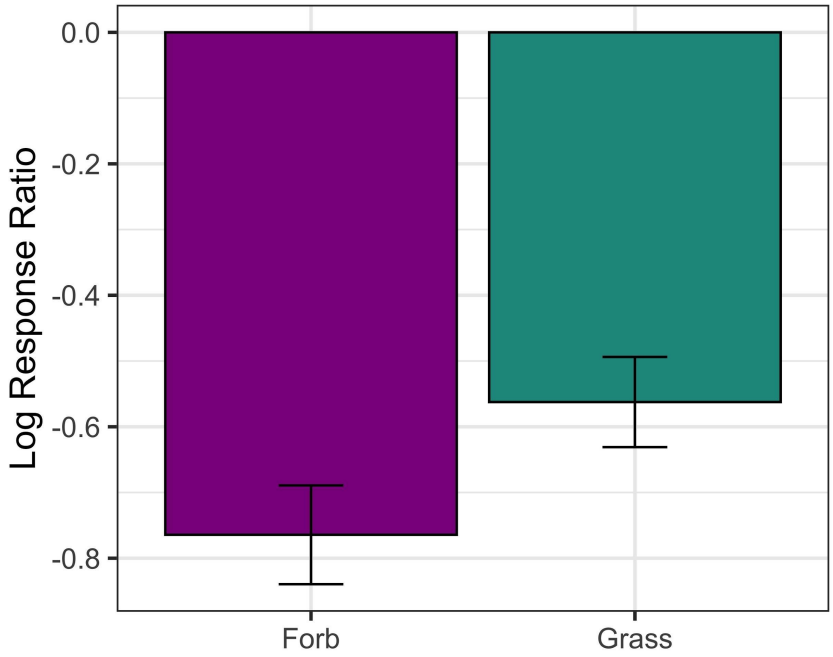

Figure 6. Bacterial families contributing to competitive outcomes. Linear mixed effects models assessing the contribution of normalized relative abundance of bacterial families to competitive outcomes (log response ratios) for forbs (purple) or grasses (green). Solid line indicates significance ($p < 0.05$), dashed line indicates nonsignificance.

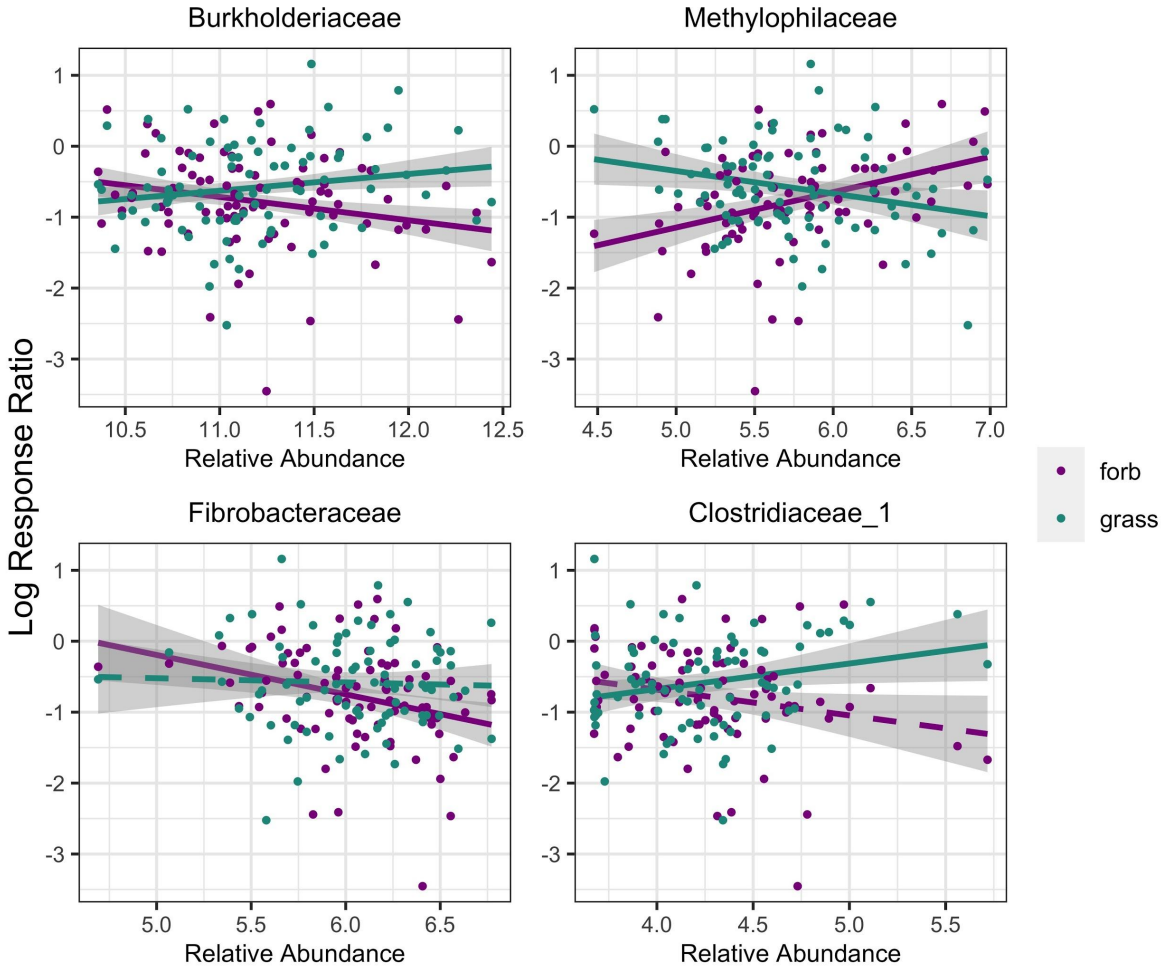
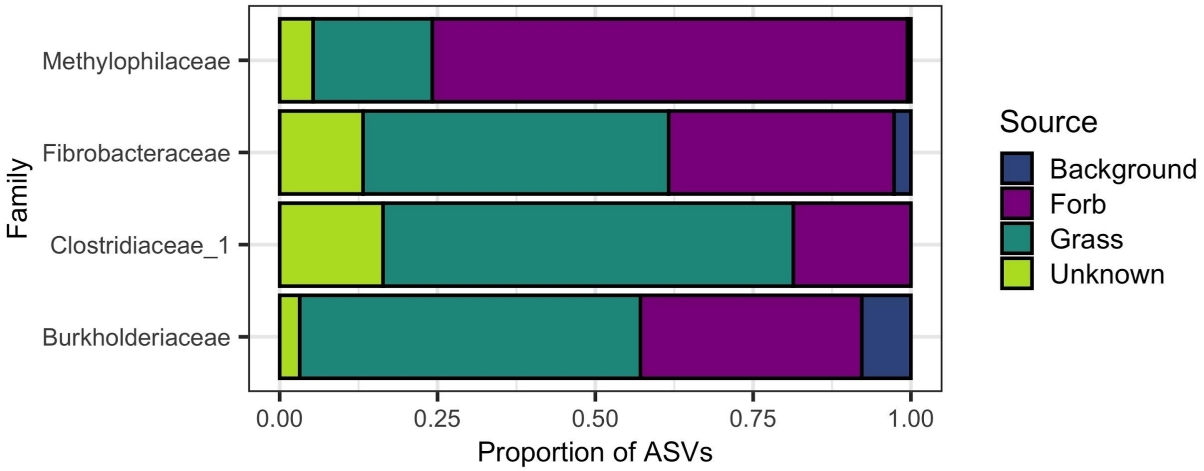
Figure 7. Proportion of ASVs predicted to colonize from each source for each of the four differentially abundant families that were correlated with competition. SourceTracker was used to predict whether ASVs in competition treatments originated from grasses (dark green), forbs (purple), the background soil mix used in the experiment (blue), or unknown habitats (light green) and then the results were summarized by grouping ASVs by taxonomic family. Families that were negatively correlated with forb competitive response and/or positively correlated with grass competitive response were predominantly sourced from grass. Methylophilaceae, the one family that showed a positive relationship with forb competitive response and a negative relationship with grass competitive response, was predominantly sourced from forbs.