Multi-Generation Ecosystem Selection Of Rhizosphere Microbial Communities Associated With Plant Genotype and Biomass In *Arabidopsis thaliana*

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Abstract

The role of the microbiome in shaping the host phenotype has emerged as a critical area of investigation, with implications in ecology, evolution, and host health. The complex and dynamic interactions involving plants and their diverse rhizosphere microbial communities are influenced by a multitude of factors, including but not limited to soil type, environment, and plant genotype. Understanding the impact of these factors on microbial community assembly is key to yielding host-specific and robust benefits for plants, yet remains challenging. Here we ran an artificial ecosystem selection experiment, over eight generations, in *Arabidopsis thaliana* L*er* and Cvi to select soil microbiomes associated with higher or lower biomass of the host. This resulted in divergent microbial communities, shaped by a complex interplay between random environmental variations, plant genotypes, and biomass selection pressures. In the initial phases of the experiment, the genotype and the biomass selection treatment have modest but significant impacts. Over time, the plant genotype and biomass treatments gain more influence, explaining ~40% of the variation in the microbial community composition. Furthermore, a genotype-specific association of a plant growth-promoting rhizobacterial taxa, *Labraceae* with L*er* and *Rhizobiaceae* with Cvi, is observed under selection for high biomass.

Keywords: plant-associated microbiome; microbial diversity; soil health; sustainable agriculture

Introduction

The conventional understanding of the host phenotype involves genetics and environment shaping observable traits. Yet, the last two decades have underscored the microbiome's significance in shaping the host phenotype, driven by extensive research on its role in ecology, evolution, and host health. The plant microbiome represents a rich source of functional diversity that is not encoded within the host genome. The interactions between the plant host and its microbiome are dynamic and reciprocal, with the plant shaping its immediate environment by exuding specific metabolites, thereby promoting the growth of specific microbial taxa, while the microbiome in turn influences plant health and growth [1]. A multitude of biotic and abiotic factors influence the dynamic nature of the microbiome, including but not limited to plant root exudates [2–4], soil type [5–9], environment [10], and various aspects of the plant, including species [11], genotype [4,12–15] and developmental stage [16,17].

Previous studies in *Arabidopsis thaliana* have shown that the microbial diversity of the soil reduced with proximity to the endophytic compartment, and microbes found in the bulk soil were not enriched in the endophytic compartment and vice-versa [18–20]. Furthermore, the microbiome of greenhouse-grown plants was similar to that of the field-grown plants, in the different plant compartments – rhizosphere, woody stem, and endophytic compartment, indicating an active role of the plant host in creating and maintaining an environment where certain microbes have improved fitness [21]. The soil environment was a stronger predictor of rhizosphere microbiome structure, though plant genotype had a weak influence in some studies [20,22,23]. A follow-up study confirmed that variation in microbiome communities depends more on the environment than on different *Arabidopsis* varieties and sister species. Though there were some differences between

47 different host species, the core microbiome interacting with the plant host at the root endophytic
48 zone was largely consistent and reproducible [24].

49 Unfortunately, most prior studies were conducted in a single plant growth cycle, precluding
50 an analysis of the temporal stability of the plant-microbiome association. Observations from plant-
51 soil feedback studies highlight that plant-microbiome interactions can be altered by successive
52 growth cycles of a given plant in the same soil [25,26]. Prior studies have employed plant-mediated
53 selection on the soil ecosystem over multiple generations, and resulted in consistent effects on
54 plant characteristics such as biomass, flowering time, and germination [27,28]. By selecting for
55 soils where hosts show the desired phenotype, such as high biomass or altered flowering time, it
56 is possible to enrich microbes that modulate host traits. Additionally, studies showed the plant's
57 response to abiotic stress, such as drought and salt stress, could be influenced in two ways. First,
58 through generational selection, where plants with desirable stress responses were chosen over
59 multiple generations [29–31]. Second, by introducing beneficial microbes that were associated
60 with plants and had previously lived in similar drought or salt conditions [32]. Mueller et al., [29]
61 used generational selection to create microbial communities that could improve plant seed
62 production by up to 205%. Soil inoculum obtained from drought-exposed soils improved wheat
63 biomass under drought conditions [32]. Older studies, such as Swenson et al. [28], also have shown
64 similar results under optimal growing conditions without necessarily exploring the microbiome. A
65 recent study conducted on the rhizosphere microbiome of wild and domesticated tomato plants
66 over multiple generations demonstrated an escalating influence of host genotype on the
67 microbiome community [33]. A separate study on the phyllosphere microbiome of various tomato
68 genotypes indicated a declining impact of host genotype across successive generations [34].

69 Despite several earlier efforts, the development of a robust and host-specific microbial
70 community with long-lasting beneficial effects on the plant host remains a challenge. Using an
71 artificial ecosystem selection experiment, we sought to advance our understanding of how plant
72 genotype, environment, and biomass selection treatment (henceforth referred to as biomass
73 treatment) impact the assembly of host microbial communities. Treatments selecting for the
74 microbial communities of high and low biomass plants did affect the growth of plants in
75 subsequent generations, though unknown environmental factors had substantial effects throughout
76 the experiment. In the first few generations of the experiment, the genotype and biomass treatments
77 played a modest role in shaping the microbial community. Over time, the plant genotype and
78 biomass treatments had increasing influence, explaining ~40% of the difference in the microbial
79 community composition. Moreover, we observe an enrichment of known plant growth-promoting
80 rhizobacteria (PGPR) in the high biomass treatment, indicating potentially beneficial host-specific
81 interactions.

82 **Material and Methods**

83 **Multi-generation selection of soil ecosystem**

84 *Arabidopsis thaliana* Cvi and L*er* accessions were cultivated using custom-made
85 "rhizotubes," (Stuart Morey, Univ. of Massachusetts, Boston, unpublished). The tubes were
86 equipped with a black polyethylene sleeve, which effectively blocked light penetration. This
87 design also facilitated the convenient removal of the plant from the pot, granting full access to
88 the root system. (Supplementary Figure S1).

89 The potting soil used was PRO-MIX PGX, a commercial mixture comprising 80-90%
90 sphagnum peat moss and small quantities of perlite. It was autoclaved twice for 40 minutes with
91 a 48-hour interval between each sterilization. The sterile potting soil was then sifted through a 3
92 mm sieve and combined with field soil in a 6:1 ratio for the first generation. The field soil

93 obtained from the Center for Agricultural Research in Waltham, Massachusetts, was composed
94 of 44% sand, 49% silt, and 7% clay representative of agricultural and grassland ecosystems.
95 The mixture was homogenized using a custom-made cement mixer and attached with
96 sterile bins to avoid cross-contamination. Distilled water was gradually added to achieve a final
97 ratio of 1:2 (water to soil). The soil was incubated at room temperature for two days following
98 inoculation before planting the seeds. All seeds used in the experiment were obtained from a
99 single-parent plant. Before planting, the seeds were treated with a solution of 50% bleach vol/vol
100 and a drop of tween 20 for 10 minutes and rinsed ten times with sterile distilled water. We placed
101 3-4 seeds in the center of each pot and kept only one seedling per pot after emergence. All plants
102 were grown at 22 ºC day/ 18 ºC night with a 12/12 hour day-night cycle in a controlled growth
103 chamber. Relative humidity ranged from 35-60% and the light intensity was 96 µE. Fertilizer
104 was not applied to plants.
105 In the first generation of the experiment, 100 plants of each accession (Cvi and L*er*) were
106 grown separately in individual pots. The plants were arranged in a randomized block design to
107 avoid batch effects. All plants were harvested 35 days after germination. The above-ground
108 portion of the plant, the rhizosphere, and the bulk soil from each rhizotube were separated,
109 ensuring there was no cross-contamination (Supplementary Figure S1). The above-ground part of
110 the plant, stem, and leaves were dried at 70 ºC for 4 days. All the plants were weighed
111 individually on a closed weighing scale accurate to 1 mg. The root-soil complex (comprising the
112 rhizosphere and the endosphere, henceforth referred to as the rhizosphere) was obtained by
113 shaking the excess soil off the root and placing the root and remaining attached soil in a sterile 5
114 ml tube. Bulk soil samples were moved to a sterile Ziplock bag. Tubes and bags were
115 immediately transferred to dry ice and then stored at -80 ºC for DNA analysis.

116 All subsequent generations were inoculated using the previously collected bulk soil from
117 the top five and bottom five plants by above-ground biomass. This resulted in two different
118 treatments for each genotype: high biomass L*er*, low biomass L*er*, high biomass Cvi, and low
119 biomass Cvi. This process is repeated for the remaining seven generations. Starting with the third
120 generation, plants were grown in uninoculated sterile potting soil, as a control for random
121 environmental variation (REV). In generation 6 all plants died for unknown reasons. The
122 experiment was then restarted using soil from generation 5. Soil sourced from the top 6-10 plants
123 and the bottom 6-10 plants based on above-ground biomass was used as inoculum to replant
124 generation 6.
125
126 **DNA extraction and 16S rRNA amplicon library prep**
127 Microbial DNA was isolated from the frozen rhizosphere samples using the Machery-
128 Nagel Nucleospin Soil DNA extraction kit (MACHEREY-NAGEL Inc., Allentown, PA, USA).
129 Approximately 0.1g of rhizosphere soil sample was used for DNA isolation. All samples were
130 diluted to 5 ng ul-1 with PCR-grade water. 16S rRNA gene was amplified from the isolated
131 DNA samples in triplicate in 96-well PCR plates. The PCR primers used were for the 16S rRNA
132 V4 region, 515F (5'-GTGYCAGCMGCCGCGGTAA-3') (ref. 35) and 806R (5'-
133 GGACTACNVGGGTWTCTAAT-3') (ref. 36) for downstream paired-end Illumina (Illumina,
134 Inc., San Diego, CA, USA) barcoded sequencing [37]. The PCR cycling conditions were as
135 follows: 94 ^{o}C for 3 min; 25 cycles of 94 ^{o}C for 45 s, 50 ^{o}C for 60 s, 72 ^{o}C for 90 s, and final
136 elongation at 72 ^{o}C for 10 min. The triplicate amplified samples were pooled and then purified
137 and normalized using the SequalPrep™ Normalization Plate Kit (Invitrogen Corporation,

138 Carlsbad, Canada). Finally, multiplexed paired-end sequencing was carried out in the Illumina
139 MiSeq (SY-410-1003) platform using earth microbiome project (EMP) primers [38].
140
141 **Sequence Data Analysis & Statistics**
142 The paired-end sequences obtained from the Illumina MiSeq were demultiplexed with
143 QIIME 2 and converted into individual sequence fastq files for each sample [37,39]. The rest of
144 the sequence processing was carried out in R (Version 4.2.2) using the DADA2 package
145 (Version 1.24.0) (ref. 40). The reads were processed in R using the following command in
146 DADA2; `filterAndTrim(fnFs, filtFs, fnRs, filtRs, truncLen=c(145,145), minLen=50, maxN=0,
147 maxEE=c(2,2), truncQ=2, rm.phix=TRUE, compress=TRUE, multithread=TRUE). `
148 *De novo* OTU (Operational Taxonomic Units) picking was performed using DADA2 (37) which
149 resolves amplicon sequence variants (ASVs) to single nucleotide differences. Chimeras were
150 removed. The taxonomy was assigned using a 100% cut-off for species-level identification with
151 the (2022) GTDB 16S rRNA reference database [41]. The phylogenetic tree was constructed
152 using IQTree [42], with 1000 ultrafast bootstraps and Modelfinder to identify the best model. In
153 this study, the best-fit model was SYM+R10 chosen according to BIC. The data comprising the
154 OTU table, phylogenetic tree, taxonomy table, and sample meta-data were then parsed through R
155 studio using the phyloseq package [43]. Here, it was processed to remove: OTUs identified as
156 mitochondrial or chloroplast, which had less than five reads across all samples, and samples that
157 had fewer than 8000 reads. The packages phytools, phyloseq, microbiome, and ggplot2 were
158 used for further data analysis. All sequence data and metadata can be downloaded from
159 PRJNA1010286.
160

Diversity Analyses

Alpha diversity was computed using four metrics, Observed, Shannon, Chao1, and Simpson diversity indices. Beta diversity was calculated using weighted and unweighted UniFrac, and subsequently Principle Coordinate Analysis (PCoA) plots were constructed using the first two axes that explain the most variance. Linear mixed effect models using, *lmerTest* package in R were used to determine changes in alpha and beta diversity over time (lmer (DiveristyMetric, Genotype*Generation*Biomass Treatment, (1|Generation)), with generation as a fixed and random effect. Pairwise comparisons were determined with *emmeans* and adjusted p-value obtained with Tukey.

Adonis

PERMANOVA was carried out for each generation using the adonis2 test. Both weighted and unweighted UniFrac distance matrices were used to account for the abundance, presence/absence, and phylogeny of the microbiome. The following model was used `adonis2(distance.matrix~Biomass Treatment+Genotype, data = meta, permutations=999)`.

Neutral Model

To determine the impact of neutral processes like drift and dispersal or by deterministic selective forces such as plant genotype and biomass treatments on microbial community assembly, we carried out the analysis as described in Burns et al. [44], which fits the data to the neutral model for Prokaryotes from Sloan et al. [45]. The following design was used, sncm.fit (spp=generation(n)_Biomass Treatment, pool = generation(n), stats=T), where n is the nth generation and 'Biomass Treatment' is high or low biomass samples.

184 *Differential Abundance*

185 There are several challenges in estimating differentially abundant (DA) taxa in
186 microbiome data, these include high variability in abundance, zero-inflated data, and the
187 compositional nature of the data. Nearing et al. [46] compared several methods across 38
188 datasets and found that different methods often identified different sets of DA taxa. The DA taxa
189 are estimated using ANCOMBC2 [47], a conservative and robust approach. ANCOMBC2
190 incorporates bias correction, effectively addressing sampling-specific and sequencing biases
191 present in the data. This feature ensures that the analysis is not skewed by any systematic errors
192 introduced during the sampling or sequencing process. It also conducts a sensitivity analysis for
193 the pseudo-count addition to assess the impact of different pseudo-count values on zero counts
194 for each taxon. The analysis was run on L*er* and Cvi samples, from OTU to Family taxonomic
195 levels with (fixed effect = (Biomass Treatment + Genotype + Generation), group = Biomass
196 Treatment).

197

198 **Results**

199 **Plant Biomass**

200 The ecosystem selection experiment was carried out for the trait plant biomass on two
201 different accessions of *Arabidopsis thaliana,* L*er* and Cvi for eight generations. The two selected
202 treatments were high and low above-ground biomass. The absolute values for the mean biomass
203 of plants (n=50) from each genotype and treatment changed substantially through the course of
204 eight generations of the experiment. Despite the stochastic fluctuations in biomass caused by
205 random environmental variation (REV), the high biomass selected lines were always the same or
206 greater than those of the low selected biomass for both genotypes (Figures 1a and 1b). Due to the

207 significant drop in biomass between generations 1 and 2, a sterile potting soil uninoculated
208 control was planted in all subsequent generations to serve as a reference for REV. The
209 uninoculated control shows that the drastic drop in biomass after generations 1 and 2, was not a
210 result of the inoculated soil but other subtle but crucial environmental factors that could not be
211 held constant. Above-ground biomass for each generation in terms of deviations from the mean
212 was plotted to give a clearer representation of the difference in phenotype seen in every
213 generation (Figures 2a and 2b). Significant differences between the low and high biomass
214 treatments become apparent from generation 4 onwards. Plants of all treatments in generation 6
215 died resulting in a sharp dip, which necessitated repeating that generation (described in the
216 Materials and Methods).

217 **Microbial community Composition**

218 After preprocessing the 16S rRNA metagenome amplicon reads in DADA 2, a total of
219 1,897,732 reads, with an average of 12822 reads per sample were obtained. Singletons and
220 chimeras were removed during pre-processing. The most well-represented phyla were
221 *Proteobacteria* (36.2%), *Bacteroidetes* (10.3%), *Planctomycetes* (7.8%), and *Actinobacteria*
222 (7.6%) (Supplementary Figure S2). The most well-represented Classes identified in the dataset
223 were *Alphaproteobacteria* (24.7%), *Gammaproteobacteria* (11.4%), *Bacteroidia (10.1%)*, and
224 *Verrucomicrobiae* (6.2%) (Figure 3). The relative abundance is typical of microbiome data,
225 heavily weighted to a few abundant groups with a long tail of rare taxa.

226 **Alpha Diversity**

227 We used two metrics to assess alpha diversity (Figure 4). Observed ($F_{7,160}$ =333.054,
228 $P<0.001$) estimates species richness, and showed a statistically significant decrease in the
229 number of OTUs (1477 to ~580) and richness across generations. The Shannon diversity index

230 ($F_{7,160}$ =82.23, P<0.001), which is more sensitive to the difference in abundance, also showed
231 this steady decrease across generations. A negative exponential function was fitted to the
232 Observed metric (R2=0.81, P<0.01) and the Shannon metric (R2=0.58, P<0.01). The Observed
233 metric displayed a better fit, indicating increased stability of the microbiome in later generations.
234 In addition, a linear mixed-effect model shows significant interactions between
235 genotype:biomass treatment (F = 8.71, P < 0.01), genotype:generation (F =3, P < 0.01), and
236 genotype:biomass treatment:generation (F = 3, P < 0.01), for Shannon and (Supplementary
237 Table S5). No significant interaction terms were found for Observed, which does not consider
238 the abundance of OTUs in its calculations.

239 **Beta Diversity**

240 Principal coordinate analyses (PCoA) of the weighted UniFrac distances were plotted
241 with the first two axes that captured the most variance in the data, from 73% in generation 1 to
242 greater than 90% in generation 8 (Figure 5). UniFrac distance considers the evolutionary
243 relationships between taxa, which makes it more biologically meaningful than other distance
244 metrics that do not consider the evolutionary history. Weighted UniFrac is more sensitive to
245 differences in the abundance of taxa. The microbiomes associated with the treatments and
246 genotypes are very similar in generation 1, but begin to diverge by generation 2. By generation 3,
247 the microbiomes of the L*er* and Cvi genotypes have diverged and by generation 5 the high and
248 low biomass treatments appear to have further distinguished the microbial communities. The
249 divergence of the microbial community in generations 4 and 5 caused by the high/low biomass
250 treatments also aligns with the onset of notable differences in the above-ground plant biomass
251 within these generations.

252 The complex interplay between genotype and biomass treatments was modeled using a
253 PERMANOVA test with both weighted and unweighted UniFrac distances to account for
254 differences in abundance and presence-absence of OTUs respectively. The resulting R^2 values
255 for genotype and biomass treatment and residuals as a proxy for REVs were plotted (Figure 6a
256 and 6b). For both weighted and unweighted UniFrac, the variance in microbial community
257 explained by both genotype and biomass treatment increased significantly over the eight
258 generations. Despite observing a decrease in the variance accounted for by the residuals, which
259 could potentially signify a decline in the influence of REVs, they accounted for ~50 percent of
260 the variability within the microbial community in generation 8. For all metrics, weighted
261 UniFrac exhibits more fluctuations compared to the unweighted UniFrac. Weighted UniFrac
262 analyses found that genotype ($F_{1,39}$=1.55, P<0.05) and biomass treatment ($F_{1,39}$=1.33, P<0.001)
263 explained 5.7% and 13.7%, respectively, of the dissimilarity between microbiomes in generation
264 1. By generation 8, genotype ($F_{1,14}$=5.14, P<0.05) and biomass treatment ($F_{1,14}$=5.48, P<0.001)
265 explain 24% and 22%, respectively, of the dissimilarity between microbiomes (Supplementary
266 Tables S6 and S7). For unweighted UniFrac, the influence of genotype on the microbial
267 community increased from 3.3% in generation 1 ($F_{1,39}$=1.33, P<0.01) to 26% in generation 8
268 ($F_{1,39}$=5.13, P<0.001), and for biomass increased from 3.8% in generation 1 ($F_{1,14}$=1.55, P<.001)
269 to 13% in generation 8 ($F_{1,14}$=2.58, P<0.05) (Supplementary Tables S8 and S9).

270 **Sloan Neutral Model**

271 Many forces can alter microbial community structure and dynamics and in our study
272 these can be divided into selective forces such as the plant genotype being colonized and the
273 biomass selection process, or neutral, stochastic forces innate in any experiment and
274 environment. The Sloan neutral model was fit to the data to assess the importance of selective

275 versus neutral drivers of change in the experiment. If neutral processes were the driving force in
276 the microbial community assembly, the null hypothesis would be that all generations of the
277 experiment would fit the model equally well. The model fit to neutrality represented by the R^2
278 value decreases from 0.795 in generation 1 to 0.59 in generation 8 demonstrating the increasing
279 importance of selective drivers through the course of the experiment (Figure 7).

280 **Differential Abundance**

281 Differentially abundant (DA) taxa that distinguished between the high and low biomass
282 treatments were determined using ANCOMBC2 at the Family level. Taxa lacking classification
283 at the Family level were denoted by the subsequent identifiable taxonomic tier. The OTUs that
284 were present in higher (red) and lower (blue) abundance in the high biomass treatment are
285 identified (Figure 8a and 8b). Among the DA taxa in this study, several are known to benefit
286 plants. The Class *Bacilli* promote plant growth [48]. The Class *Gemmatimonadetes* are
287 positively associated with vegetation restoration, plant richness, and soil nutrients [49].
288 *Cytophagacea* are chemoorganotrophs and important for remineralizing organic materials into
289 micronutrients. They could support both mycelial growth and plant nutrition [50].

290 *Enterobacteriaceae*, *Paenibacillaceae*, and *JACDCH01* were all present in higher
291 abundance in the high biomass treatments of both L*er* and Cvi. Most *Paenibacillaceae* members
292 predominantly inhabited soil, frequently in close association with plant roots [51]. These
293 rhizobacteria were known to play a significant role in enhancing plant growth and possessing
294 potential applications in agriculture and *Enterobacteriaceae* had been reported to enhance plant
295 growth [52–54].

296 Additionally, *Order_NS11-12g*, *BJHT01*, *JACDCH01*, and *UBA6156* were more
297 abundant in Cvi under high biomass conditions and less abundant in L*er* under high biomass
298 conditions. The majority of these were uncultivated or candidate taxa. Two alternative families
299 in the Order *Rhizobiales* appeared differentially associated with the two plant genotypes:
300 *Labraceae* with L*er* and *Rhizobiaceae* with Cvi [55]. These could indicate genotype-specific
301 interactions with different members of the microbial community.
302 The relative abundance of all OTUs that exhibited higher abundance (enriched) in
303 samples from the high biomass treatment in the L*er* and Cvi is depicted in Figures 8 (c) and 8 (d)
304 respectively. These OTUs consistently exhibit an increasing trend in relative abundance across
305 successive generations. A linear model was employed to test this trend, resulting in R-squared
306 values of 0.57 ($P < 0.01$) for L*er* and 0.73 ($P < 0.01$) for Cvi. Furthermore, OTUs that were
307 lower in abundance (depleted) in samples from the high biomass treatment in L*er* and Cvi are
308 depicted in Figures 8 (e) and 8 (f) respectively. These OTUs persist in low abundance across all
309 generations, suggesting a lack of selective pressure on this group of microbes in the samples
310 from the high biomass treatment.
311
312 **Discussion**
313 We applied artificial ecosystem selection for eight generations, in *Arabidopsis thaliana*
314 L*er* and Cvi to select soil microbiomes associated with higher or lower biomass of the host. In
315 contrast to some previous studies [27,33], we did not fertilize plants thus maintaining nutrient
316 limitation, and thereby promoting the plant's interaction with the microbiome [56]. Over the
317 course of eight generations, a response to the selection, most apparent after generations 4 and 5,
318 was observed both in the microbiome characteristics that shifted gradually and in the plant

319 biomass. Microbiome selection noticeably influenced plant biomass despite large phenotypic
320 variation from one generation to the next. Stochasticity in growth through generations may be
321 attributed to random environmental variations (REVs) as indicated by Swenson et al. [28]. This
322 was observed in the uninoculated sterile potting soil reference that showed similar patterns of
323 variability as the inoculated treatments across generations (Figure 1). Ecological variability
324 among generations even under controlled growth chamber conditions is a common characteristic
325 of studies [57] and may be due to minute fluctuations in growth chamber conditions or batch and
326 age of potting soil.

327 We observed a gradual decline in microbial species richness during the initial generations
328 of the experiment. This was evident from the observed OTU counts, as well as the Chao1 and
329 Shannon diversity indices. This pattern is consistent with findings from other studies [32–34],
330 highlighting the impact of selection pressures as the microbial communities adapted to the host
331 plant environment. However, in our study which continued for about twice the number of
332 generations of these earlier studies, we observed a stabilization of richness and alpha diversity in
333 the later generations. This was further substantiated by the stronger fit to the negative exponential
334 function when compared to a linear model, indicating constant and proportional changes in alpha
335 diversity and the rhizosphere microbiome's trend towards reduced diversity loss over time,
336 accompanied by heightened stability over eight generations.

337 To further understand the complex interplay between the forces of directed selection (plant
338 genotype and biomass treatment), we did a PCoA. Over successive generations, the results
339 demonstrate a strengthening effect of plant genotypes and biomass treatment on the microbial
340 community (Figure 5). This result was modeled using PERMANOVA, for both weighted and
341 unweighted UniFrac distances. Both weighted and unweighted UniFrac show marked increases in

342 the proportion of variance explained by genotype and biomass treatment, with unweighted UniFrac
343 exhibiting larger increases from generation to generation (Figure 6). Both metrics consider
344 phylogenetic relatedness. Weighted UniFrac considers the absolute abundance of OTUs and is
345 generally used to study changes in microbial community structure. Unweighted UniFrac considers
346 only the presence/absence of OTUs and is generally used to study changes in microbial community
347 composition. The results suggest that variance in microbiome composition as explained by
348 genotype consistently increases, whereas variance in microbiome structure as explained by
349 genotype, fluctuates more with changes in abundant taxa.

350 Here we would like to highlight that pronounced shifts in biomass between the high/low
351 biomass treatments, alpha diversity, and beta diversity all occur around generations 4 and 5 (Figure
352 1 and 2). This marks the point at which the changes in alpha diversity stabilize (Figure 4) and the
353 divergence between microbial communities by high/low biomass treatments in the PCoA plots
354 becomes more pronounced (Figure 5). It is possible that over the first four to five generations, the
355 starting microbial community underwent a period of restructuring before it stabilized and formed
356 four distinct communities under selection by high/low biomass treatments and by plant genotype.
357 We propose a complex interaction between plants and their associated microbiome ensued, where
358 differences in root exudation patterns between the two genotypes presumably established
359 associations with microbes in the early generations. Simultaneously, during this period, the
360 biomass treatment likely promoted host-specific microbe-mediated interactions that modulate
361 plant biomass. This restructuring of the microbial community is driven by both structural
362 (abundance of OTUs) and compositional (presence/absence of OTUs) effects in the microbiome
363 (Figure 6a and 6b). Differences in the abundance of taxa driven by the biomass and genotype

364 selection pressures, was a larger contributor to the generation-to-generation variation during the
365 experiment.

366 Changes in the assembly of microbial communities can arise from either selective pressure,
367 like the plant genotype or biomass treatment observed in this experiment, or stochastic processes,
368 like minute changes in growth chamber humidity or microenvironment. To gain deeper insights
369 into the influence of selection on microbial community assembly, a neutral model was fit to the
370 data [44,45]. Interestingly, this revealed a progressive decline in fit to neutrality, indicated by
371 decreasing R^2 values and increasing AIC values (Figure 7). This suggests an increasing influence
372 of selective forces such as biomass treatment and genotype over multiple generations of ecosystem
373 selection. This was also previously observed by Morella et al. [34].

374 Previous studies in *Arabidopsis thaliana* have often detected only a weak genotypic effect
375 on the rhizosphere microbiome [23,24,58]. However, a majority of these studies are focused on a
376 single growth cycle. Insights from plant-soil feedback research emphasize that the interactions
377 between plants and their microbiomes can be modified by consecutive growth cycles of the same
378 plant species in its respective soil [25,26]. Recent research has presented conflicting findings on
379 the influence of host genotype [33,34,59]. The different experimental designs, communities being
380 assessed and the length of these experiments, alongside high stochastic environmental variation
381 that is clearly associated with these microbial studies likely account for the conflicting findings.
382 In our investigation, we find that during the initial stages, genotype and biomass treatments have
383 modest but significant impacts. Over time, the plant genotype and biomass treatments have an
384 increasing influence. Together these selective forces explained ~40% of the variation in the
385 microbial community composition in these later generations.

386 A key aim of microbiome engineering has been to develop host-specific microbial
387 communities that impart lasting beneficial effects on the plant host [27,29,32,60]. Here we show
388 the enrichment of some common taxa in high biomass treatments but also some genotype-specific
389 changes. Despite starting with the same soil, only three common families were enriched in the
390 high biomass treatment samples of both the L*er* and Cvi genotypes. In addition, well-known plant
391 growth-promoting rhizobacteria were enriched in a genotype-specific manner for both L*er* and Cvi
392 high biomass treatment samples. In the Order *Rhizobiales*, *Labraceae* was enriched in L*er,* while
393 *Rhizobiaceae* was enriched in Cvi [55]. Moreover, in high biomass conditions, Cvi had increased
394 levels of the taxa Order_NS11-12g, BJHT01, JACDCH01, and UBA6156, while L*er* had
395 decreased levels of these. These data suggest genotype-specific plant genetic control of the
396 rhizosphere microbiome.

397 To further explore how the abundance of these OTUs changed across successive
398 generations, we grouped all the DA OTUs that were enriched or depleted in the samples of high
399 biomass treatment and calculated the sum of their relative abundance. The OTUs enriched in the
400 samples of high biomass treatment consistently displayed an increasing trend in relative
401 abundance over subsequent generations (Figures 8c and 8d). A linear model was fitted to this
402 data with high R^2 values, reinforcing the notion that the genotype and biomass treatments exerted
403 selective pressure on entire microbial groups, rather than individual species. Conversely, the
404 OTUs that were depleted in the samples of high biomass treatment consistently remained in a
405 state of low relative abundance throughout the entirety of the experiment (Figures 8e and 8f).
406 This observation may indicate that the selective pressures exerted by the genotype and biomass
407 treatments do not influence this particular group of microbes.

408 Taken collectively, these findings suggest the presence of diverse selection pressures
409 influences the rhizosphere microbiome. Initially, it seems that neutral processes seem to play a
410 major role in determining the structure, and composition of the microbiome (Figure 7). As
411 generations progress, we observe an increase in the strength of directional selection, possibly
412 driven by genotype and biomass treatment, resulting in the enrichment of specific groups of
413 microbes, including some PGPRs (Figures 8c and 8d). The persistence of depleted microbes in
414 high biomass treatment samples suggests the possibility for negative frequency-dependent
415 selection, preserving them at a low abundance. On the other hand, it is possible that this group is
416 experiencing no observable selection pressure.

417 Multiple studies have shown variations in the structure of the rhizosphere microbiome,
418 even within closely related plant genotypes, highlighting the importance of genotype-specific root
419 exudates in forming associations with the corresponding microbiome [61–65]. Further exploration
420 must prioritize highlighting the mechanisms that uphold these interactions through the utilization
421 of comparative metagenome, metatranscriptome and metaproteome analyses. This will play a part
422 in decoding chemical crosstalk, which could eventually foster interactions between plants and
423 microbes, leading to an overall enhancement in plant fitness.

424 The relationship between plants and microorganisms in the rhizosphere involves complex
425 and diverse interactions, which influence crucial ecological and physiological processes. These
426 interactions can be mutualistic, competitive, or antagonistic in nature. Many biotic and abiotic
427 factors act in concert to influence the dynamic rhizosphere microbiome. Gaining insights into how
428 these factors influence the assembly of microbial communities is crucial for obtaining targeted and
429 long-lasting advantages for plants. Nevertheless, establishing a strong and host-specific microbial
430 community that consistently provides beneficial effects continues to be a persistent challenge. We

431 have shown that, despite stochastic fluctuation due to REVs, it is possible to select for microbial
432 communities that impact biomass in a host-specific manner within four generations, addressing
433 this key challenge in microbial community engineering [32,60]. The rhizosphere microbiome that
434 evolves under plant-mediated selection offers better chances of survivability and efficacy when
435 applied as inoculum to the plant [66,67]. Multi-species ecosystem selection provides a perspective
436 on microbiome assembly that indicates the holobiont, and the evolving relationship between host
437 and microbiome contributes to emerging properties beyond those predicted by host genotype or
438 initial microbial community composition. This study enhances our understanding of the temporal
439 dynamics involved in microbial assembly in the rhizosphere and has implications for sustainable
440 agriculture, evolution, and ecology.
441

FIGURES

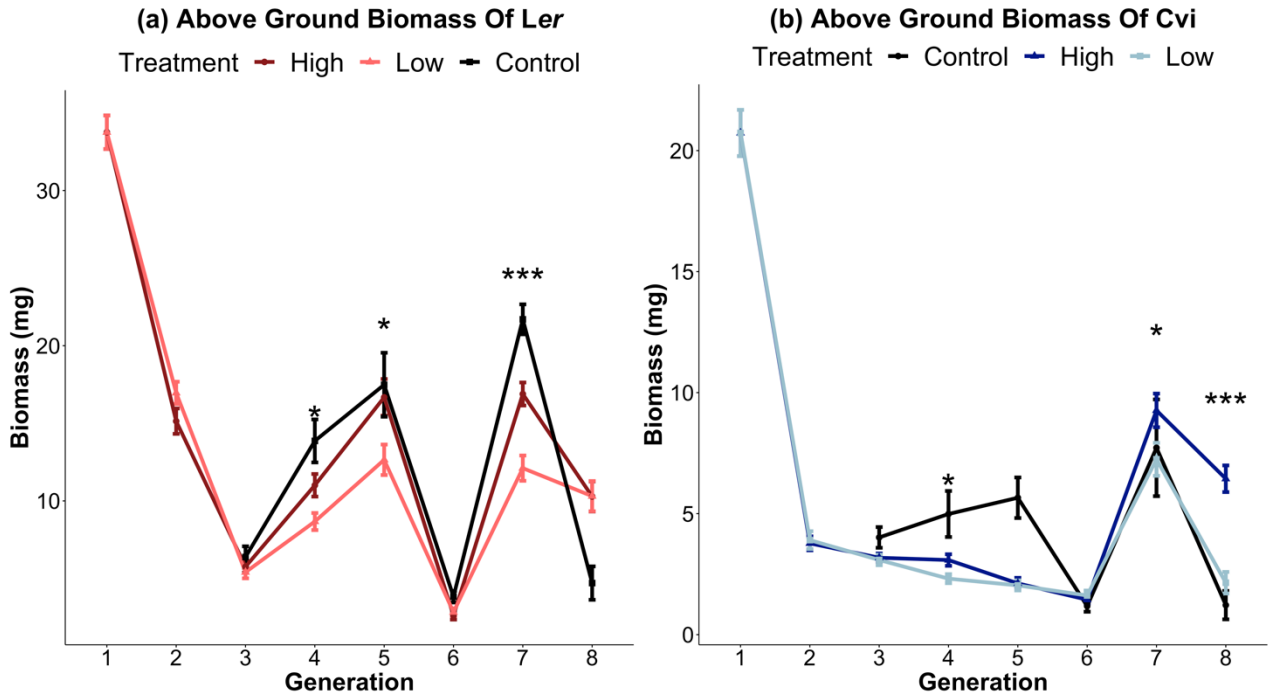

Figure 1. Ecosystem selection leads to changes in above-ground plant biomass. Above ground biomass for genotypes Ler (a) and Cvi (b) of *Arabidopsis thaliana*. Dark lines represent the high biomass treatment, and light lines represent the low biomass treatment. Each point represents the mean of n=50 microcosms. The mean biomass of the selection lines differed for several generations of both genotypes (t.test p-value * < 0.05; **<0.01;*** <0.001).

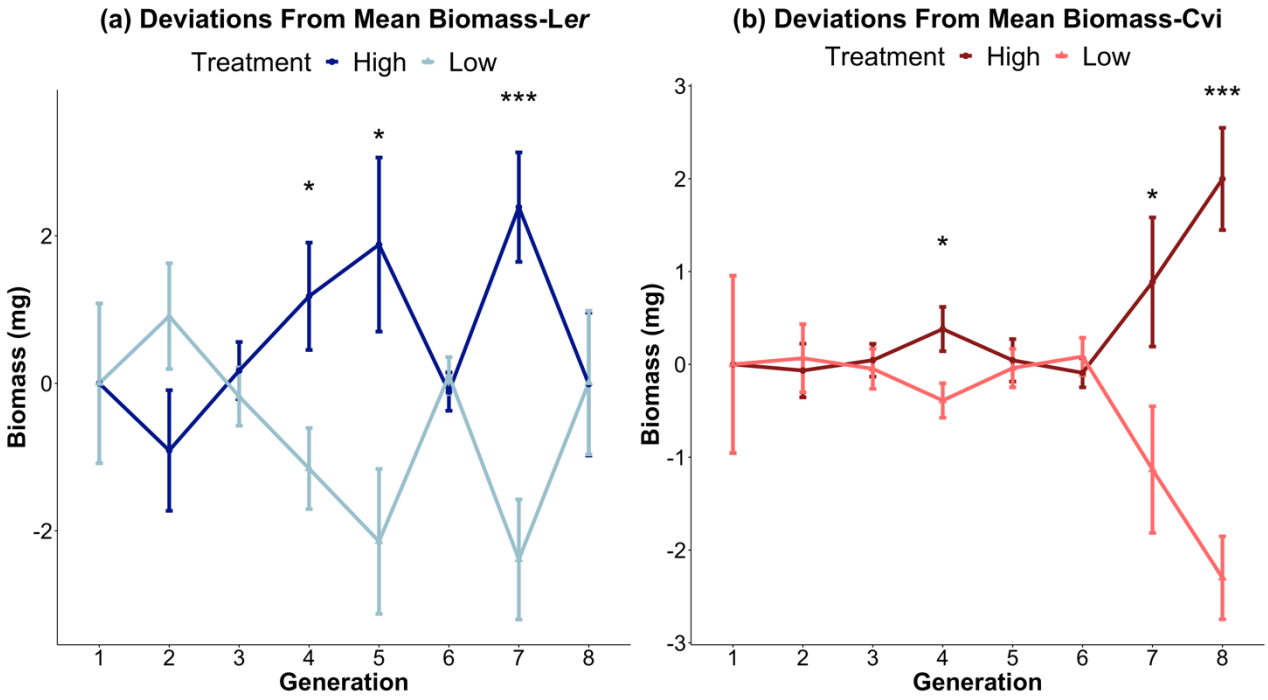

Figure 2. Above ground biomass represented as deviations from means for each generation. By plotting deviations from the mean, the influence of REVs on above-ground biomass is mitigated for genotypes L*er* (a) and Cvi (b). The dark lines denote high biomass treatments, while the light lines represent low biomass treatments. Each data point is the average of n=50 microcosms. (t.test p-value * < 0.05;**<0.01; *** <0.001).

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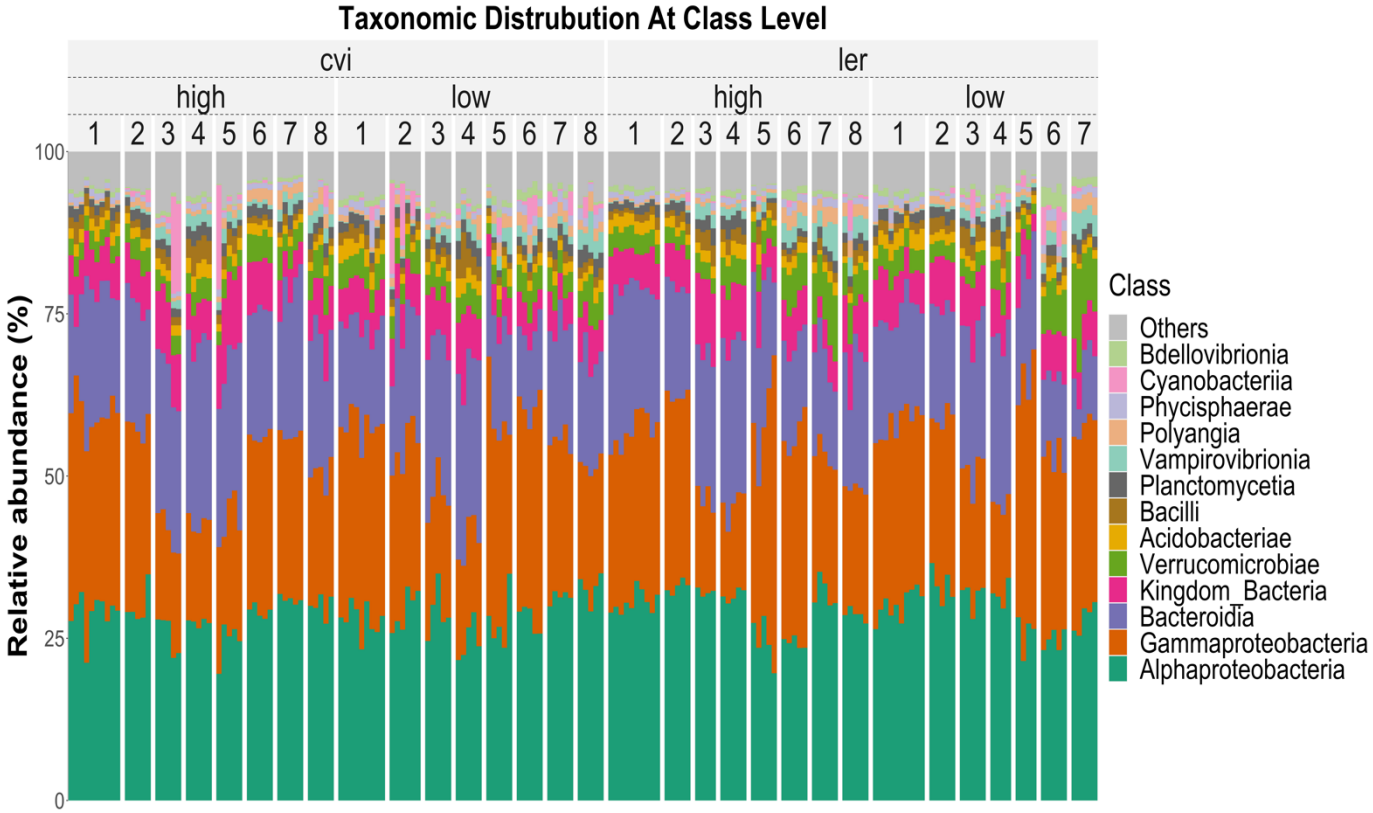

Figure 3. Taxonomic distribution of the microbial community is represented in terms of the relative abundance at the Class level. The plot shows eight generations (1-8) for the high and low biomass treatments of both the Cvi (left) and L*er* (right) genotypes of *Arabidopsis thaliana*. Ten samples were assayed for generation 1 experiments while five were done for all subsequent generations. The most represented classes across all samples were *Alphaproteobacteria* (24.7%), *Gammaproteobacteria* (11.4%), *Bacteroidia* (10.1%), and *Verrucomicrobiae* (6.2%).

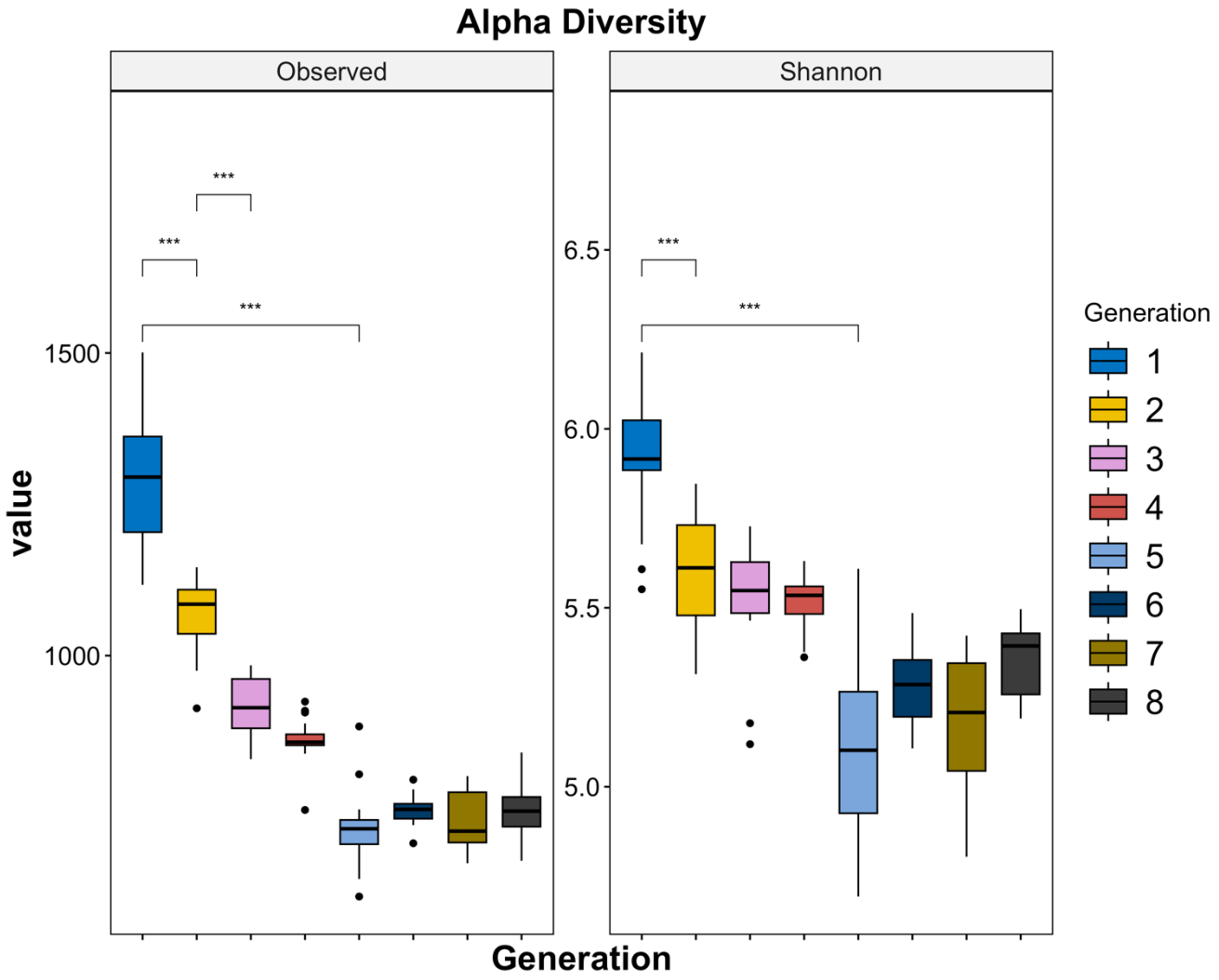

Figure 4. Changes in alpha diversity. Over the course of eight generations, two alpha diversity indices were computed, including the Observed and Shannon metrics. Results indicate a significant decline in the Observed number of OTUs, which estimates species richness. The Shannon index, which considers species abundance also exhibited a noteworthy decrease from generation 1 to 8. Additionally, a negative exponential function was fit to the Observed (R^2=0.81, P<0.01) and Shannon (R^2= 0.58, P<0.01) metrics, with the Observed showing a better fit indicating increased stability of the microbiome in later generations. (wilcox test *<0.05, **<0.01, *** <0.001).

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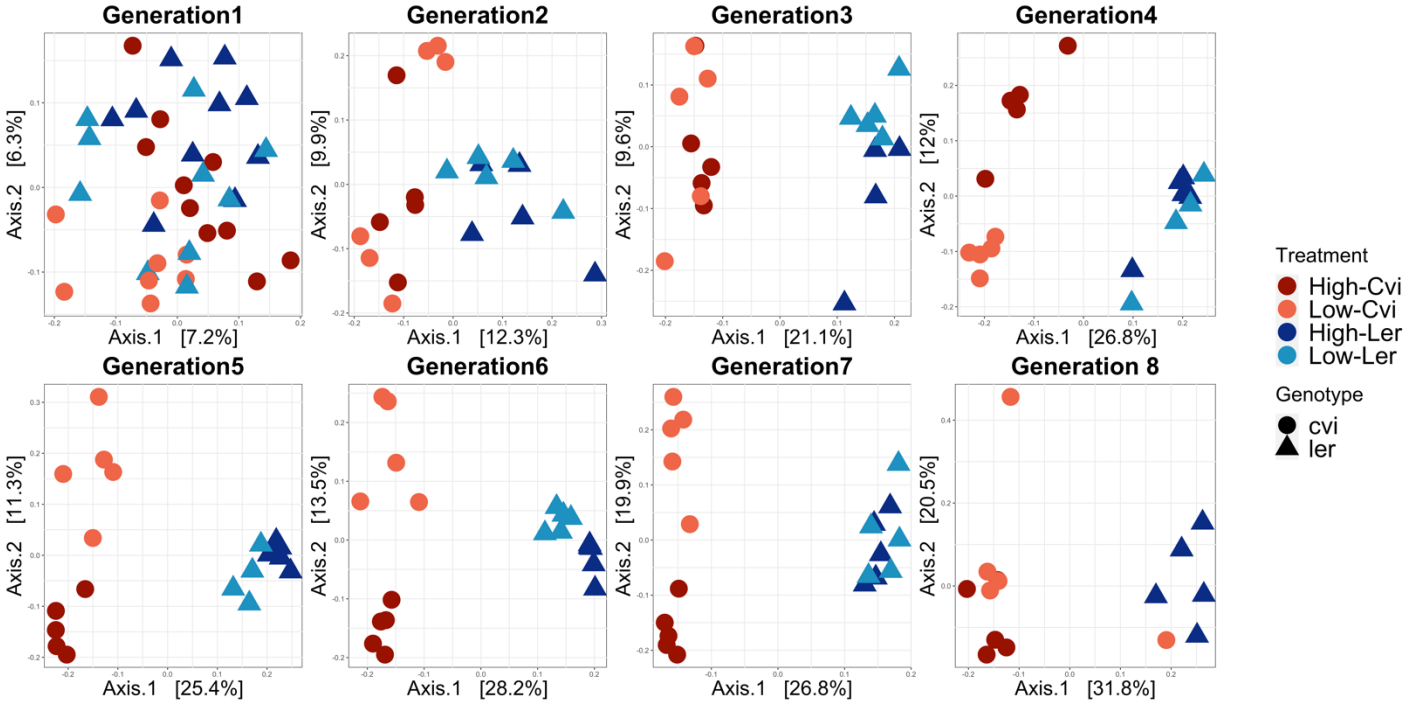

Figure 5. Host genotype and biomass treatment shape the composition of the microbiome. Principle Coordinate Analysis (PCoA) using unweighted UniFrac for generations 1 through 8. Each point represents an individual sample with microbial communities defined of Cvi represented by red/orange circles and L*er* by dark/light blue triangles. The first two coordinate axes plotted accounted highest variation in the data, ranging from 13% in generation 1 to approximately 52% in generation 8. While there was no discernible clustering in generation 1, both genotypes and high/low biomass treatments exhibited greater clustering over the course of the experiment.

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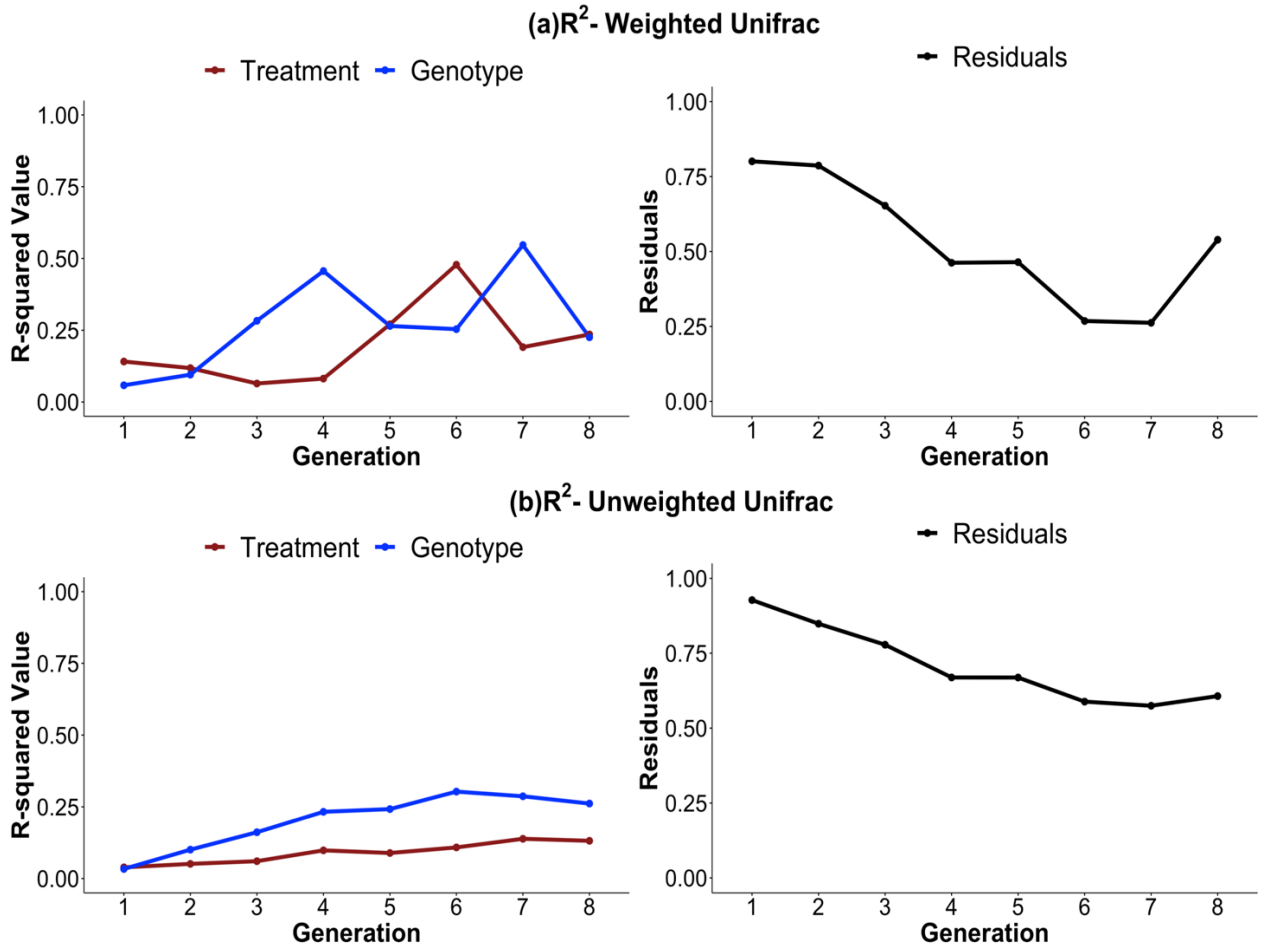

Figure 6. Variance in microbiome structure and composition explained by genotype and biomass treatment increases over generations. The R^2 values were computed using the PERMANOVA test with the adonis2 function for generations 1 through 8 for (a) weighted and (b) unweighted-UniFrac distances. The resulting R^2 and variance explained by residuals values for the biomass treatment (brown), genotype (blue) and residuals (black) are plotted. Residuals are plotted as a proxy for random environmental variations (REVs). There is a marked increase in the influence of both genotype and biomass treatment on differences in the microbial community from generations 1 through 8. Despite observing a decrease in the variance accounted for by the residuals, which could potentially signify a decline in the influence of REVs, the model elucidates more than 50 percent of the variability within the microbial community in the 8th generation. These differences are more pronounced in the weighted vs unweighted UniFrac. Model (distance.matrix~biomass treatment +genotype).

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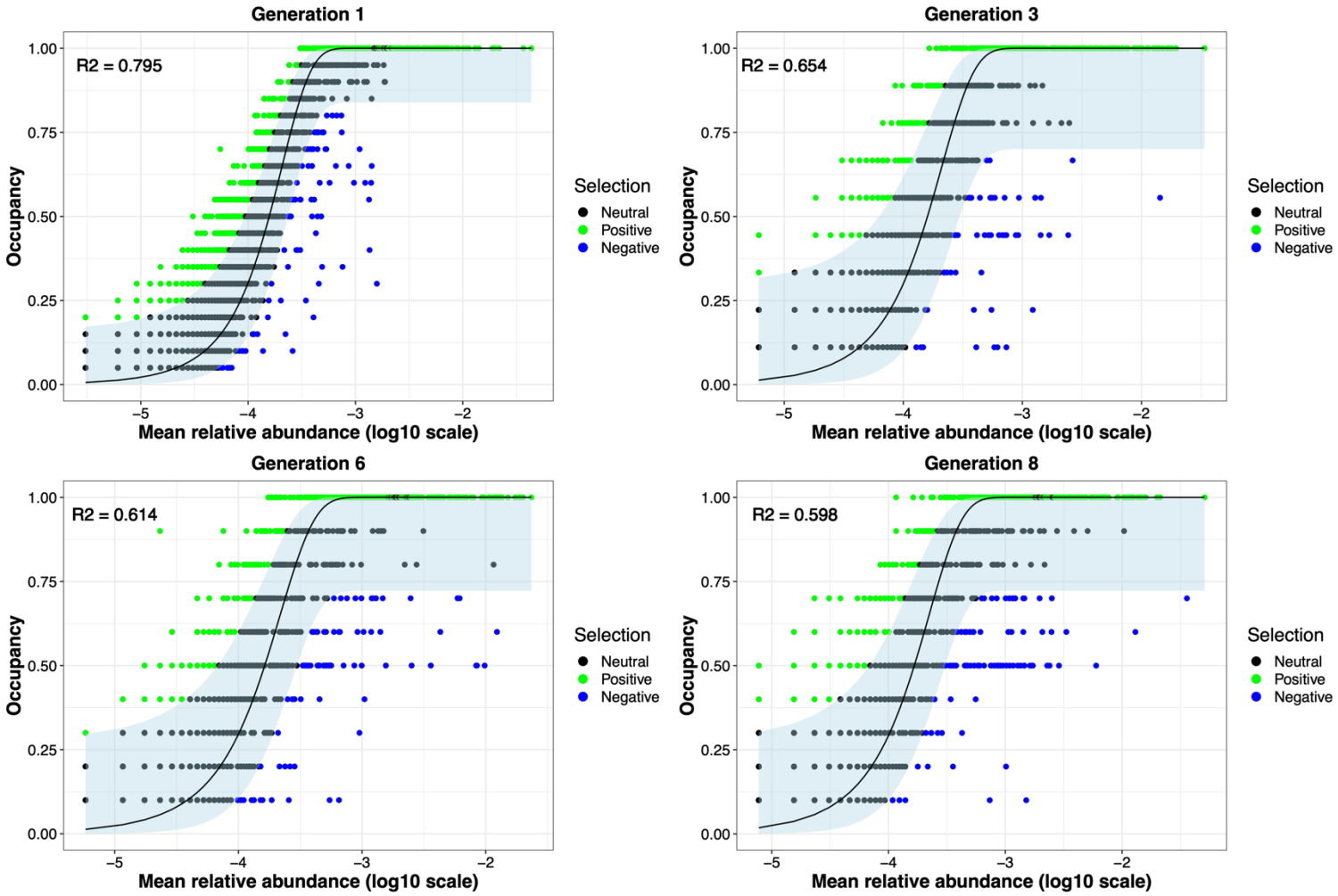

Figure 7. Fit of the Neutral model diminished as generations progressed. The occupancy (prevalence of OTU in samples) plotted against log(10) abundance is depicted using the Burns model. OTUs that are neutral, i.e., not selected for or against, are indicated in black. Green and blue-colored OTUs signify positive and negative selection, respectively. This plot highlights the presence of numerous microbes that undergo directed selection throughout the experiment.

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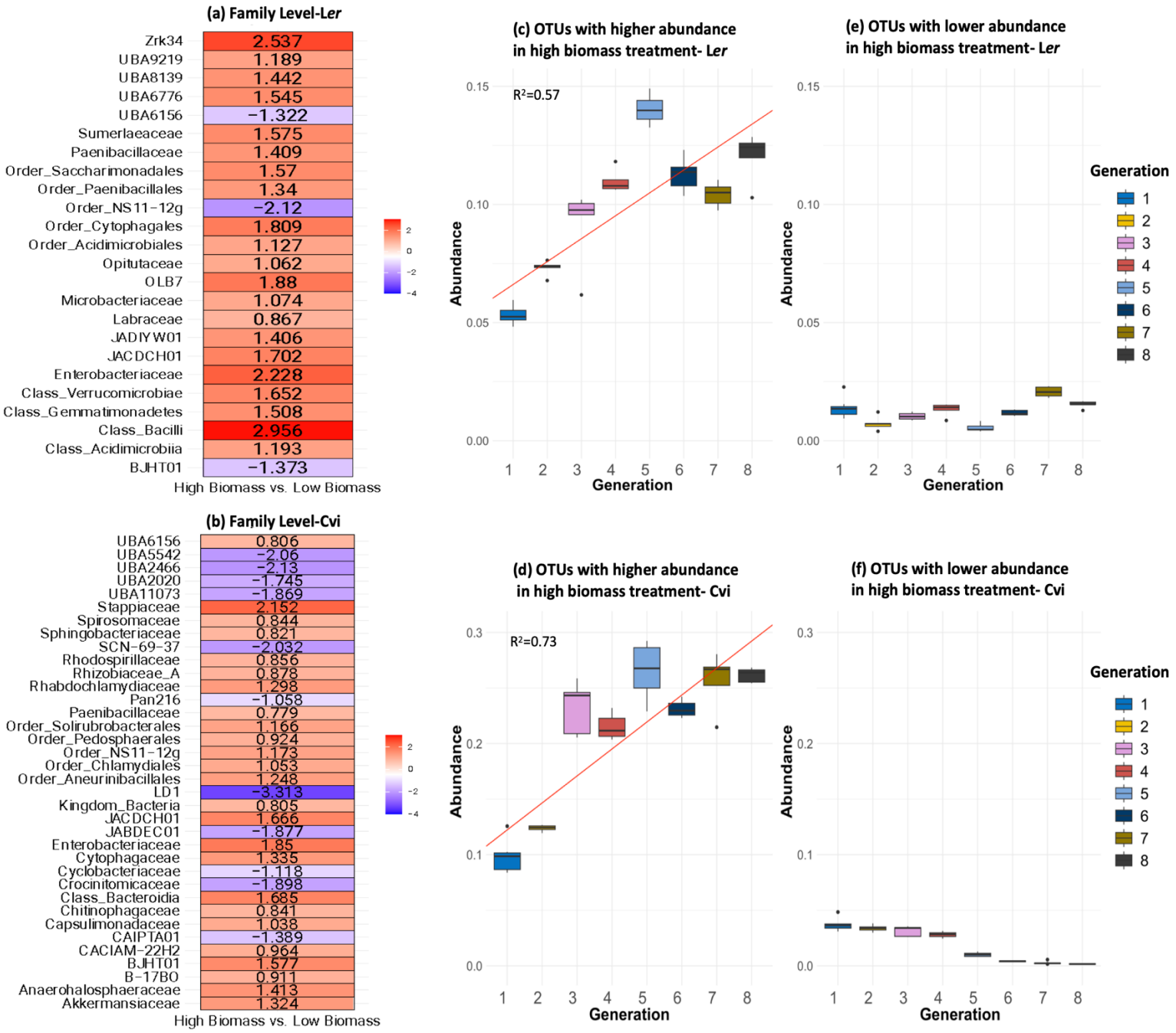

Figure 8. Differentially abundant OTUs. ANCOMBC2 results are illustrated as $\log_2$ fold changes at the family level for high biomass vs low biomass samples. Each taxon listed in the figure represents a single OTU in either the L*er* (a) or Cvi (b) microbial community identified to the family level if possible. The scale indicates $\log_2$ fold change, red and blue for positive and negative fold change in the high biomass samples respectively. Well-known plant growth-promoting bacteria, such as *Paenibacillaceae, Bacilli, Labraceae, Rhizobiaceae* and *Bdellovibrio* are present in higher abundance. A vast majority of the other taxa identified are uncultivated and have little to no information on them. Figures (c) and (d) show box plots of relative abundance for all OTUs that were enriched in samples of the high biomass treatment in

genotype L*er* and Cvi, respectively, with colors indicating different generations. These OTUs demonstrate a consistent trend of increasing relative abundance across successive generations. To test this trend (red line), a linear model was fitted to the data, resulting in an R-squared value of 0.57 ($P < 0.01$) for L*er* and 0.73 ($P < 0.01$) for Cvi. Figures (e) and (f) are box plots of relative abundance for all OTUs that were depleted in samples from the high biomass treatment in genotypes L*er* and Cvi, respectively, and colored by generation.

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519 **Supplementary Materials:** Figure S1: Rhizotubes schematic; Figure S2: Taxonomic
520 distribution of the microbial community represented in terms of the relative abundance at the
521 Phylum level. Table S1: Statistical comparisons of biomass for L*er*; Table S2: Statistical
522 comparisons of biomass for Cvi; Table S3: ANOVA for Observed alpha diversity index; Table
523 S4: ANOVA for Shannon alpha diversity index; Table S5: Linear mixed effect model for
524 Shannon diversity index; Table S6: PERMANOVA weighted UniFrac distances for generation 1;
525 Supplementary Table S7: PERMANOVA weighted UniFrac distances for generation 8;
526 Supplementary Table S8: PERMANOVA unweighted UniFrac distances for generation 1; Table
527 S9. PERMANOVA unweighted UniFrac distances for generation 8.
528

529 **Author Contributions:** N.S. Conceptualization, methodology, data analysis and visualization,
530 data collection, writing-original draft, writing-reviewing and editing. P.S. Methodology, data
531 analysis, writing-original draft, writing-reviewing, and editing. T.M. Data collection. R.K.
532 Resources, funding, writing-reviewing, and editing.
533

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