Population bottlenecks constrain host microbiome diversity and genetic variation impeding fitness

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Abstract

It is becoming increasingly clear that microbial symbionts influence key aspects of their host's fitness, and *vice versa*. This may fundamentally change our thinking about how microbes and hosts interact in influencing fitness and adaptation to changing environments. Here we explore how reductions in population size commonly experienced by threatened species influence microbiome diversity. Consequences of such reductions are normally interpreted in terms of a loss of genetic variation, increased inbreeding and associated inbreeding depression. However, fitness effects might also be mediated through microbiome diversity, such as through loss of functionally important microbes. Here we utilise *Drosophila melanogaster* lines with different histories of population bottlenecks to explore these questions. The lines were phenotyped for egg-to-adult viability and their genomes sequenced to estimate genetic variation. The bacterial 16S rRNA gene was amplified in these lines to investigate microbial diversity. We found that 1) host population bottlenecks constrained microbiome richness and diversity, 2) core microbiomes of hosts with low genetic variation were constituted from subsets of microbiomes found in flies with higher genetic variation, 3) both microbiome diversity and host genetic variation contributed to host population fitness, 4) connectivity and robustness of bacterial networks was low in the inbred lines regardless of host genetic variation, and 5) reduced microbial diversity was associated with weaker evolutionary responses in stressful environments. These findings suggest that population bottlenecks reduce hologenomic variation (in combined host and microbial genomes). Thus while the current biodiversity crisis focuses on population sizes and genetic variation of eukaryotes, an additional focal point should be the microbial diversity carried by the eukaryotes, which in turn may influence host fitness and adaptability with consequences for the persistence of populations.

Introduction

It is becoming increasingly clear that most eukaryotes live in intimate and complex relationships with microbial communities both in their external environment and on or within their body including their gut (McFall-Ngai *et al.* 2013). Numerous studies have documented how the presence and abundance of certain microbes can influence key aspects of host fitness, such as lifespan, fecundity, immune responses, metabolic health, behaviour, and thermal stress tolerance traits (Sommer & Bäckhed 2013; Ericsson & Franklin 2015; Mueller & Sachs 2015; Sampson & Mazmanian 2015; Sison-Mangus *et al.* 2015; Moghadam *et al.* 2018; Douglas 2019). Conversely, the host can also control microbial composition to some extent, such as through changing nutrient availability by diet choice or host metabolism (Muegge *et al.* 2011; Jehrke *et al.* 2018), triggering immune factors (Douglas 2019), or controlling the gut mechanically such as through peristalsis (Coyte *et al.* 2015). The genetic background of the host can also interact with the microbiome (Chandler *et al.* 2011; Dobson *et al.* 2015). For instance, host genes can affect the abundance of certain bacteria, allowing the microbial composition of hosts to be treated as a quantitative trait in genetic analysis (Chaston *et al.* 2016). These interactions between the host and its microbiota can in turn have a substantial impact on host fitness (Gould *et al.* 2018; Walters *et al.* 2019; West *et al.* 2019).

Because interactions between microbes and their hosts shape so many aspects of life, core biological processes including evolutionary adaptation may need to be reconsidered (Shapira 2016; Koskella & Bergelson 2020; Moeller & Sanders 2020; Henry *et al.* 2021). The 'holobiont' concept reflects the idea that eukaryote individuals do not act as autonomous units, but rather as networks consisting of the host and all its associated microbiota, and that their collective genomes – the 'hologenome' – forms a single unit of selection (Zilber-Rosenberg & Rosenberg 2008; Bordenstein & Theis 2015; Kristensen *et al.* 2021). Some experimental support for this idea is emerging. For instance, host selection for thermal tolerance resulted in an altered microbial composition and modulated the microbes' response to temperature (Kokou *et al.* 2018). While resident microbes respond to stressful environmental conditions, they can also subsequently aid the response of the host to such conditions (McFall-Ngai *et al.* 2013; Moghadam *et al.* 2018). More generally, the microbial community could play a so-far

underappreciated role in the broader context of population persistence which is important for research areas like conservation biology (Bahrndorff *et al.* 2016; Shapira 2016; Trevelline *et al.* 2019; West *et al.* 2019).

These conjectures raise the issue of how microbes respond to changes in a host's population size. Populations that undergo repeated or persistent reductions in size frequently suffer from inbreeding depression and genetic drift resulting in lowered fitness and reduced genetic variations (Charlesworth & Charlesworth 1987; Kristensen & Sørensen 2005), ultimately impeding evolutionary capacity and increasing the risk of extinction (Spielman *et al.* 2004; Frankham 2005; Markert *et al.* 2010; Hoffmann *et al.* 2017; Ørsted *et al.* 2019). These patterns have been investigated from the perspective of the nuclear genetic background rather than the hologenome; for instance, inbreeding depression is normally assumed to reflect the expression of deleterious alleles in their homozygotic form. However, host population size decreases may also lead to microbial perturbations, with potential feed-back effects on host fitness which could contribute to additional lowering of population size (Redford *et al.* 2012; Bahrndorff *et al.* 2016; Trevelline *et al.* 2019), although the interacting effects of microbial diversity, genetic diversity and fitness have not yet been widely tested.

Here we investigate microbial composition and abundance in 50 populations of *Drosophila melanogaster* that have experienced a variable number of population bottlenecks. These populations were subsequently phenotyped for fitness components and sequenced to obtain a genome-wide molecular measure of genetic variation (Ørsted *et al.* 2019). This set of lines provides a unique resource to investigate associations between host genetic variation, host fitness and microbial diversity (**Fig. 1**). We consider two main questions. 1) How is microbiome richness and diversity associated with host genetic variation imposed by population bottlenecks? 2) Is both host microbiome diversity and genetic variation associated with host fitness, and if so, do they contribute independently? Answers to these questions increase our understanding of the importance of microbiomes in conservation and evolutionary genetics. We hypothesise that reduced host genetic variation provides a less diverse environment for microbes leading to reduced microbial diversity through fewer host genotype-microbial taxon-specific interactions. Evidence suggests that increased microbiome diversity is adaptive for the

host and that increased hologenome genetic variation facilitates faster evolutionary responses to selection (Hauffe & Barelli 2019; Henry *et al.* 2021). Our work further highlights the importance of considering both host and microbiome diversity for predicting evolutionary consequences of population bottlenecks in small and threatened populations.

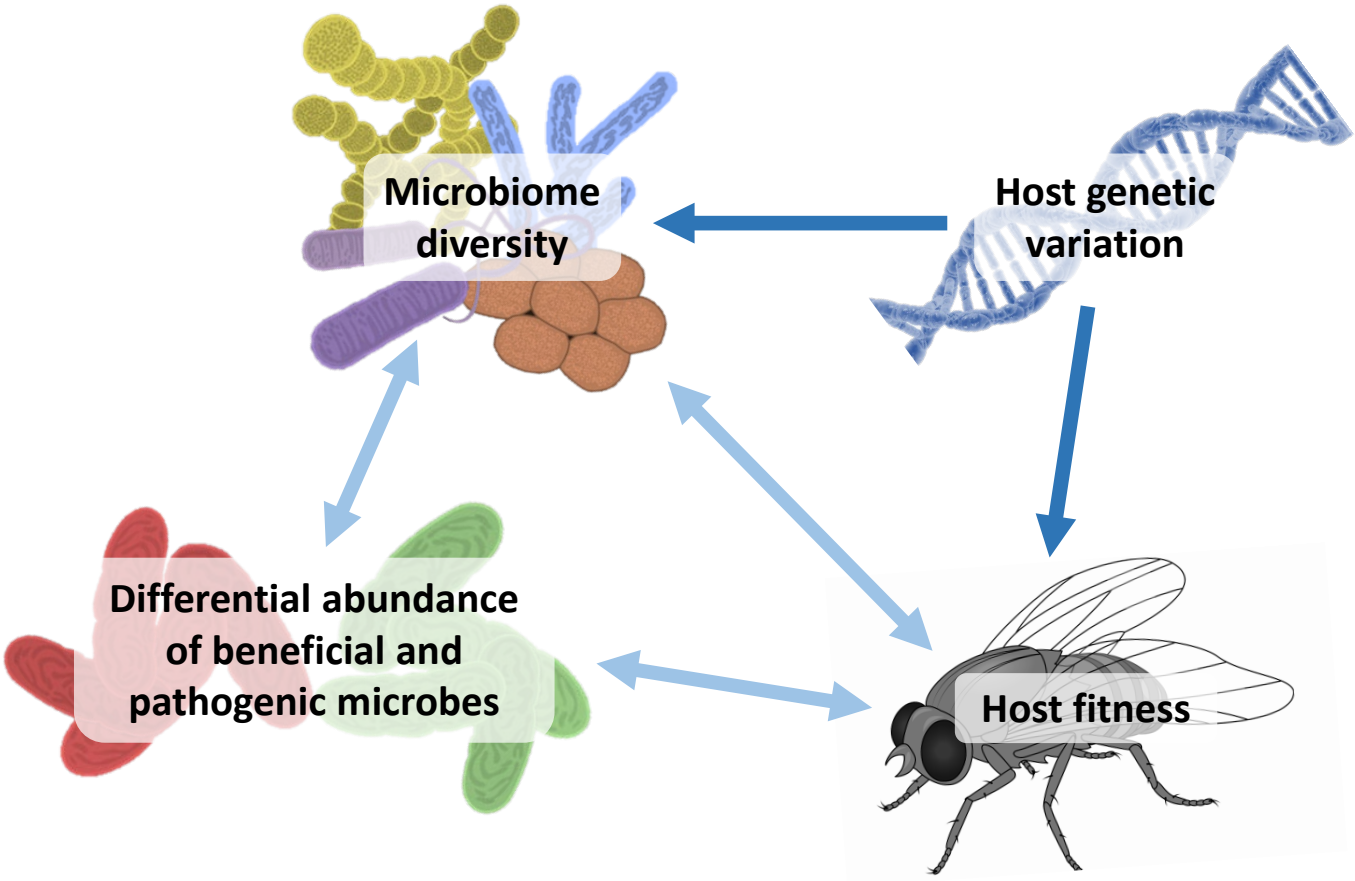


Figure 1.

Conceptual illustration of hypothetical associations between host genetic variation, microbiome diversity and host fitness in the experimental set of lines. Host genomic variation was manipulated through exposing populations to a variable number of bottlenecks and is assumed to have a causal effect on fitness and microbial diversity. The dark blue arrows represent potential unidirectional effects, and light blue arrows represent potential bidirectional effects. In one line of causality, low genetic variation in the host constrains the diversity (species richness and abundance) of the host-associated microbiome, which in turn affects host fitness directly, i.e. through supporting fewer host genotype-microbial taxon-specific interactions that are functionally important, and/or indirectly through changing the composition

107 and relative abundance of beneficial or pathogenic microbes. In another line of causality, low host
108 genetic variation directly affects host fitness which in turn leads to a less diverse microbiome through
109 physiological or metabolic changes in the host "environment", or indirectly such as through a higher
110 abundance of detrimental pathogenic microbes resulting from a weakened immune response.

111 Results

112 We selected 50 lines for microbiome analysis, which represent a subset of 109 lines that went through
113 marked reductions in population size for a varying number of generations and the 10 outbred control
114 lines kept at a large population size. These original 119 lines had been sequenced using Genotyping-
115 By-Sequencing (GBS) to allow nucleotide diversity (π) to be estimated. In addition, the fitness
116 component egg-to-adult viability (hereafter just viability) was determined in each line (see Ørsted *et al.*
117 2019 for details on genomic variation and phenotypes assessed). We found a positive correlation
118 between viability and π for these 119 lines (Spearman's ρ=0.45; p<0.001; **Supplementary Fig. S1**)
119 indicating inbreeding depression for viability which is used here as a proxy for Darwinian fitness. For
120 the microbiome characterization, two groups of 25 lines were selected based on measures of
121 standardized viability and π (sum of Z-scores, see methods). One group of 25 lines had low genetic
122 variation and low viability, and another group of 25 lines had high genetic variation and high viability.
123 The 'high' group included nine of the outbred controls as the outbred lines generally had very high
124 viability and nucleotide variability. The outbred lines and the inbred lines in the 'high' group did not
125 differ statistically in genetic variation. To distinguish between effects of genetic variation within inbred
126 lines *versus* inbreeding/outbreeding, we separated lines into three categories: 'low genetic variation'
127 and 'high genetic variation' inbred lines and 'outbred' (OB) control lines (**Supplementary Fig. S2**).
128

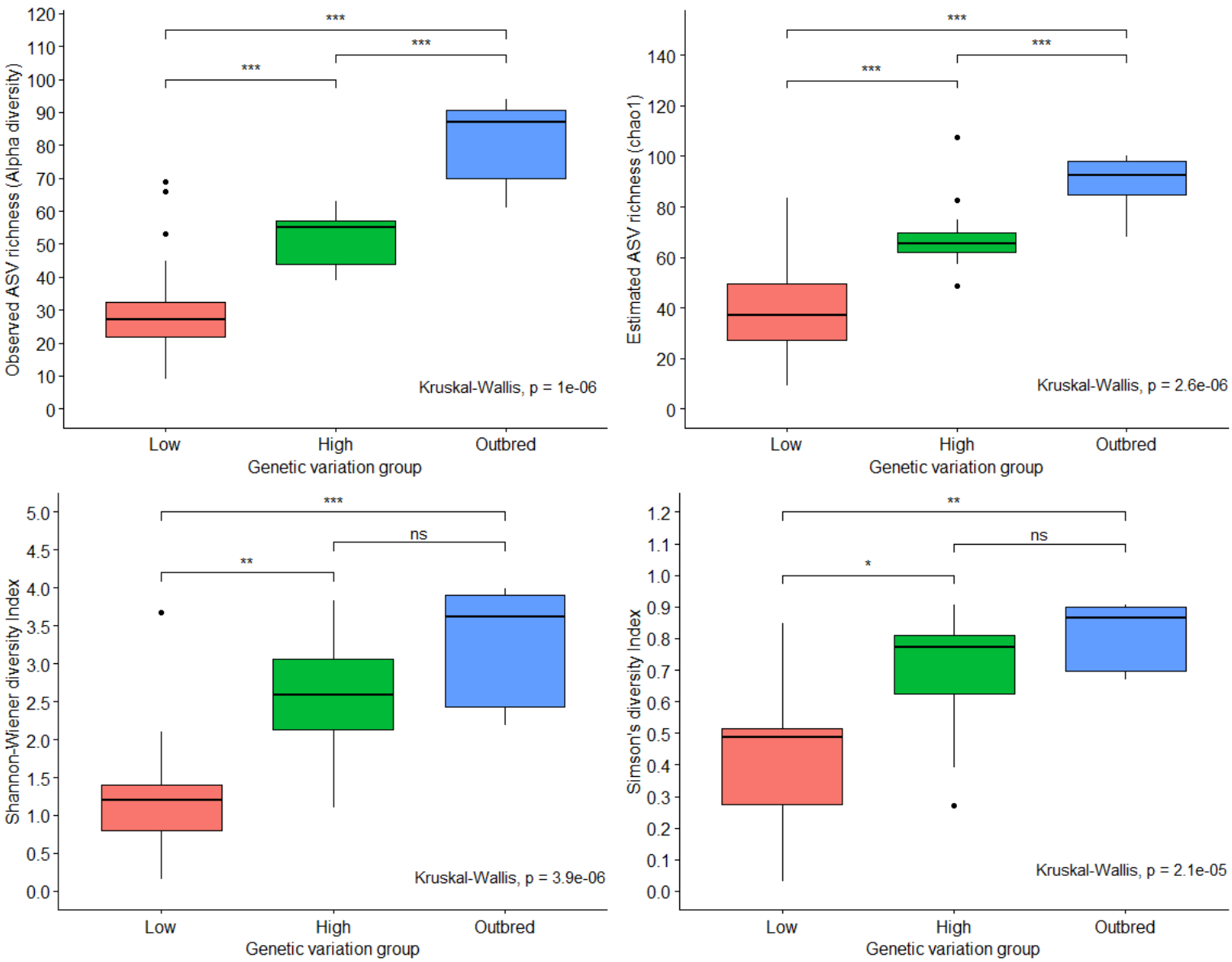
129 *Loss of host genomic variation decreases microbiome richness and diversity*

130 Loss of host genetic variation was associated with decreased microbiome richness and diversity. The
131 microbiomes from the outbred flies and the high genetic variation lines had a higher level of community
132 diversity than the microbiomes of flies from the low genetic variation group (**Fig. 2**). Generally, there

133 was an increase in microbiome diversity with increasing fly host genetic variation and viability,
134 regardless of which measure we used to quantify the microbiome diversity. Richness indices, namely
135 observed amplified sequence variant (ASV) richness and chao1 estimated ASV richness (**Fig. 2A-B**),
136 showed a stronger trend than the diversity indices accounting for relative abundances (Shannon-Wiener
137 and Simpson's; **Fig. 2C-D**). Even at the class level, the microbiomes from the low genetic variation
138 group were less taxonomically heterogeneous than those from the high genetic variation and outbred
139 groups (**Supplementary Fig. S3**). The majority of bacteria in the *D. melanogaster* microbiomes
140 belonged to the Alphaprotoebacteria, Bacilli, and to a lesser extent Bacteroidia and Actinobacteria. The
141 increasing level of taxonomic diversity with increasing host genetic variation was further evident at the
142 genus level, with the low genetic variation group harbouring the fewest number of genera, while the
143 outbred flies had the highest number (**Supplementary Fig. S4**). Non-metric multidimensional scaling
144 (NMDS) analysis based on the weighted and unweighted UniFrac distances (**Fig. 3**) showed that the
145 microbiomes differed depending on the fly host's level of genetic variation. Generally, there was better
146 segregation of the fly groups when only the microbiome membership (unweighted UniFrac) was
147 considered (**Fig. 3C-D)**, with a clear trend across the viability and nucleotide diversity gradient across
148 the first dimension of ordination space. Meanwhile the relative abundance patterns of the ASVs across
149 the samples created noise, notably among the high and outbred groups (**Fig. 3A-B)**. However, the
150 community membership was more similar among the outbred lines and more dispersed within the inbred
151 lines (**Fig. 3C-D**), while the abundance patterns showed greater similarity among the low genetic
152 variation flies than the higher variation and outbred flies, due to the high abundance of a few ASVs in
153 common in the low variation flies (**Fig. 3A-B**). PERMANOVA analysis showed that the fly groupings
154 explained 22.8% and 43.0% of the microbial community membership and structure (i.e. membership +
155 abundance using weighted UniFrac), respectively ($p < 0.001$). The host traits, nucleotide diversity and
156 viability were significantly associated with the microbiome community patterns (significant envfit
157 arrows, **Fig. 3A-D**). Viability explained a greater proportion of the microbiome composition than did
158 nucleotide diversity, where nucleotide diversity and viability explaining 15.9% and 17.8% of the
159 community membership, while explaining 27.7% and 35.3% of the community structure.

Both host genetic variation and microbiome diversity contribute to host fitness

In order to identify drivers of host fitness, we fitted generalized linear mixed effects models (GLMMs) with egg-to-adult viability as a function of host nucleotide diversity (π) and microbiome diversity and their interaction (one model for each of the four different measures of microbiome diversity; see methods). For all microbiome diversity metrics, both π and microbiome diversity contributed to host fitness, with π contributing to a greater extent (~2 x larger scaled effect sizes; **Table 1; Supplementary Table S1**). For Alpha richness, we observed a significant positive interaction between host genetic variation and microbial diversity on host fitness, meaning that they did not act independently. In fact, the positive interaction coefficients suggested synergism; i.e. in lines with high genetic variation, the effect of increasing microbiome richness was greater than in lines with low genetic variation and *vice versa* (**Supplementary Fig. S5**), while for chao1 ASV richness, Shannon-Wiener and Simpson's indices, host genetic variation and microbial diversity contributed independently to host fitness (**Supplementary Table S1)**. In all cases, the full models had higher explanatory power than each individual model (π or microbiome diversity alone), based on χ^2 tests (**Table 1; Supplementary Table S1**). Using data from Ørsted *et al.* (2019) for the set of lines used in the present study, we also associated microbial diversity with evolutionary responses in two traits (dry body mass and productivity measured as eggs per female per day) here defined as the slope of an ordinary linear regression across 10 generations of rearing on a stressful medium. Interestingly, we found an effect of microbial diversity on evolutionary responses for both traits (**Supplementary Fig. S6**). For details on assessment of these phenotypes, see Ørsted *et al.* (2019).



181 **Figure 2.**

182 Boxplots of microbiome diversity between the three genetic variation groups; low genetic variation
183 (Low; red), high genetic variation (High; green), and outbred (blue) measured as either richness indices
184 (**A**. observed ASV richness (Alpha diversity), and **B**. estimated ASV richness (chao1 estimation)) or
185 measured using indices accounting for individual ASV abundances as well (**C**. Shannon-Wiener Index,
186 and **D**. Simpson's Index). In all panels, the *p* values of a Kruskal-Wallis test show a significant effect
187 of group, while asterisks denote the results of pairwise Wilcox's t-tests between groups; *** $p < 0.001$;
188 ** $p < 0.01$; * $p < 0.05$; and ns: $p > 0.05$.

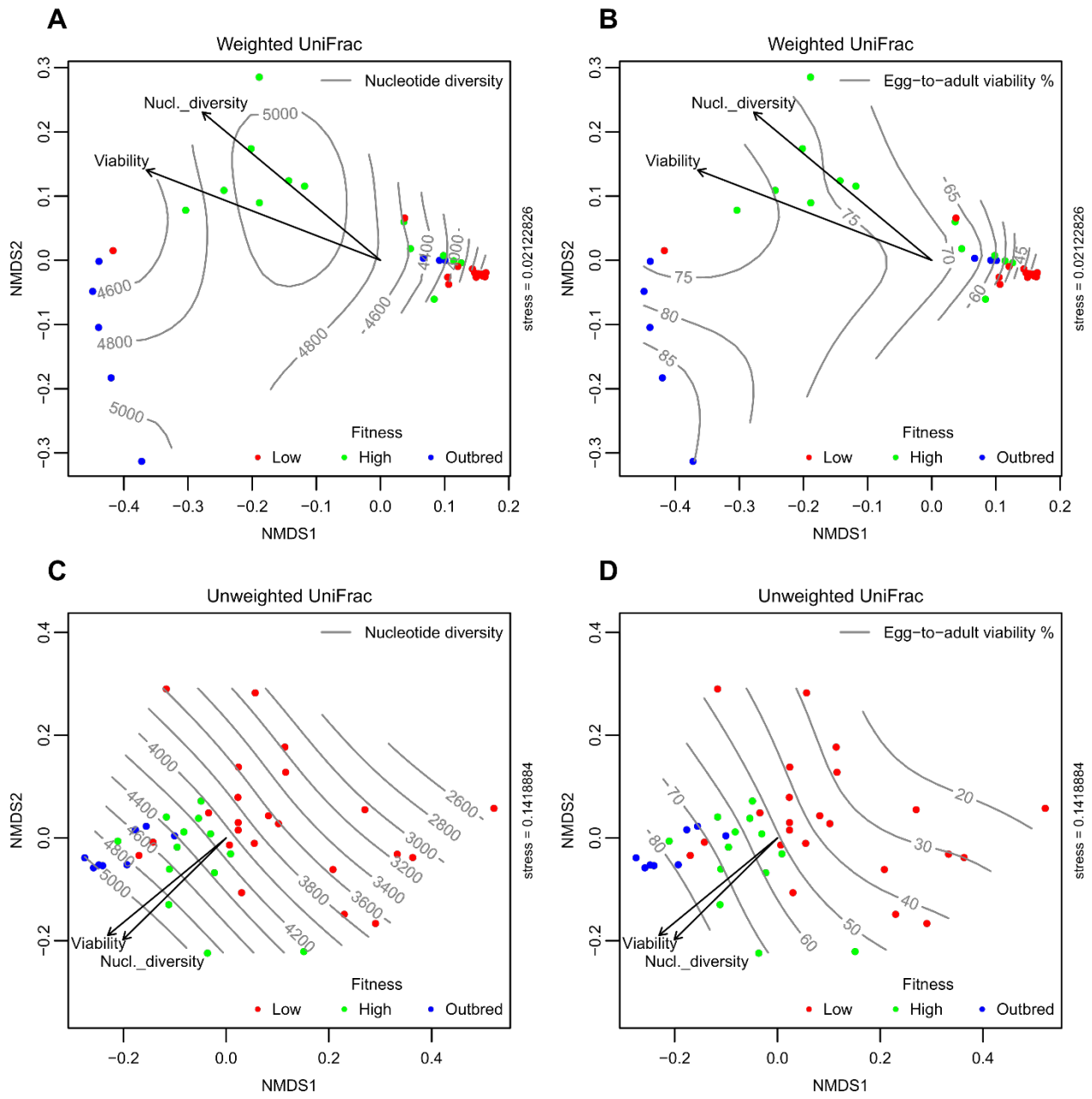


Figure 3.

Non-metric multidimensional scaling (NMDS) plots based on the UniFrac distances (weighted; **A-B**, and unweighted; **C-D**) between the 50 *D. melanogaster* lines (low genetic variation (red), high genetic variation (green), and outbred (blue). UniFrac distances account for the relative relatedness of community members, where weighted unifrac incorporates the abundance of observed organisms, while unweighted unifrac only considers presence or absence. Isolines of associated covariates are shown for nucleotide diversity (**A** and **C**) and egg-to-adult viability (**B** and **D**). Envfit values of significant drivers ($p < 0.05$) are shown as arrows (viability and nucleotide diversity, respectively).

Table 1.

Results of the general linear mixed model (GLMMs) of egg-to-adult viability as a function of nucleotide diversity (NuclDiv) and microbiome diversity (Alpha richness; ObsAlpha) and their interaction as fixed effects. Both dependent and independent variables are scaled (Z-standardization) to allow direct comparison of effect sizes. Replicate vial IDs were included as a random effect, as flies from the same vial are not considered independent. Conditional coefficients of determination of the GLMMs $\left(R^2_{\text{full model}}\right)$ interpreted as the variance explained by the entire model, including both fixed and random effects, are shown. Asterisks denote the significance of individual variables or interactions; *** $p < 0.001$; ** $p < 0.01$; and * $p < 0.05$. The full model including both dependent variables and their interaction is compared with individual models with either nucleotide diversity or alpha diversity with a χ^2 test.

Microbiome diversity measure	Fixed effects	Estimate	Std. Error	z value	p	
Observed ASV Richness (ObsAlpha)	Intercept	0.106	0.081	1.311	0.1899	
$R^2_{\text{full model}} = 0.355$	NuclDiv	0.784	0.088	8.883	6.49E-19	***
	ObsAlpha	0.499	0.087	5.740	9.49E-09	***
	NuclDiv*ObsAlpha	0.194	0.089	2.182	0.0291	*
	Random effects	**Std.Dev**				
	Vial	0.683				
	Comparison with individual models	**χ^2 df**	**χ^2**	**p**		
	NuclDiv; ObsAlpha; Full model	2	70.322	5.37E-16	***	

210 *Host genetic variation is associated with differential bacterial abundance*

211 The subset of the bacterial communities that were differentially more abundant in the outbred flies were
212 more diverse than in the inbred lines (i.e. the low and high genetic variation groups), with as many as
213 54 ASVs belonging to five bacterial classes and 12 orders (Supplementary Table S2). In comparison,
214 only one *Acetobacter* ASV was significantly more abundant in the bottlenecked flies, i.e. the low and
215 high genetic variation groups. More generally, at both the ASV and genus levels, *Acetobacter*
216 abundances were particularly high in the low genetic variation lines compared to the lines with higher
217 genetic variation (**Fig. 4A-C**), with *Acetobacter* ASVs making up the majority of the communities in
218 the low genetic variation lines (median 94.0% for Zotus 1+2 and 96.9% for the genus). In contrast, five
219 ASVs belonging to *Lactobacillus* and *Enterococcus* were significantly more abundant in the high
220 genetic variation and outbred flies, while at the genus level, *Enterococcus* but not *Lactobacillus* showed
221 this same trend (**Fig. 4 D-F**, and **Supplementary Table S2**). In addition, certain ASVs displayed
222 incremental increases in relative abundances with increasing level of genetic variation and outbreeding,
223 despite DESeq results being non-significant (**Fig. 4F-H**).

224

225 *Core microbiomes of the low genetic variation group are subsets of the high genetic variation groups*

226 The number of ASVs that were present in at least 80% of samples was defined as the core microbiome.
227 The number of ASVs belonging to this core decreased with decreasing host genetic diversity in the fly
228 groups, with 48, 22, and 11 ASVs in outbred, high, and low genetic variation flies respectively (**Table**
229 **2**). Eleven of these ASVs persisted as part of the core microbiome across all of the fly groups, and
230 belonged to the *Acetobacter*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, and Bacilli. Meanwhile, nine
231 ASVs belonging to the *Corynebacterium*, *Empedobacter*, Nocardiaceae, *Enterococcus*, *Mesorhizobium*,
232 and *Lactobacillus*, were only found in the core microbiomes of the high and outbred flies. The core
233 microbiome of the lower genetic variation lines were subsets of those present in the higher genetic
234 variation lines and outbreds, with 11/11 low genetic variation group ASVs represented in the high
235 genetic variation group, and 20/22 high genetic variation group ASVs represented in the outbred group
236 (**Fig. 5, Table 2**).

Table 2.

Core microbiome ASVs in each of the three host genetic variations groups: low genetic variation (Low), high genetic variation (High), and outbred (OB). The ASVs belonging to the core microbiome for each fly group is represented by + (core) and – (not core). Here we define presence in the core microbiome if an ASV is present in at least 80% of samples of a particular group. The lowest taxonomy level is listed (o__: order, f__: family, g__:genus).

ASVs	Class	Order	Family	Genus	Species	Low	High	OB
ASV1	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Acetobacter*	*sp.*	+	+	+
ASV2	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Acetobacter*	*sp.*	+	+	+
ASV4	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Acetobacter*	*sp.*	+	+	+
ASV5	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	*sp.*	+	+	+
ASV7	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*plantarum*	+	+	+
ASV8	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	*sp.*	-	-	+
ASV9	Bacilli	Lactobacillales	Leuconostocaceae	*Leuconostoc*	*sp.*	+	+	+
ASV10	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Corynebacterium*	*sp.*	-	+	+
ASV11	Bacteroidia	Flavobacteriales	Weeksellaceae	*Empedobacter*	*sp.*	-	+	+
ASV12	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*sp.*	+	+	+
ASV13	Actinobacteria	Corynebacteriales	Nocardiaceae	*g__*	*sp.*	-	+	+
ASV14	Actinobacteria	Corynebacteriales	f__	*g__*	*sp.*	-	+	+
ASV15	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*sp.*	+	+	+
ASV16	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*plantarum*	+	+	+
ASV17	Actinobacteria	Corynebacteriales	Nocardiaceae	*g__*	*sp.*	-	+	+
ASV18	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	*sp.*	-	+	+
ASV19	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Corynebacterium*	*sp.*	-	-	+
ASV21	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas*	*sp.*	-	-	+
ASV22	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	*Sphingobacterium*	*mizutaii*	-	-	+
ASV23	Gammaproteobacteria	Burkholderiales	Alcaligenaceae	*Alcaligenes*	*sp.*	-	-	+
ASV24	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	*Sphingobacterium*	*mizutaii*	-	-	+
ASV25	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Mesorhizobium*	*sp.*	-	+	+
ASV27	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*brevis*	+	+	+
ASV28	Gammaproteobacteria	Burkholderiales	Alcaligenaceae	*Alcaligenes*	*sp.*	-	-	+
ASV29	Actinobacteria	Corynebacteriales	Nocardiaceae	*Rhodococcus*	*sp.*	-	-	+
ASV30	Actinobacteria	Micrococcales	Microbacteriaceae	*Leucobacter*	*sp.*	-	-	+
ASV32	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*g__*	*sp.*	-	-	+
ASV36	Actinobacteria	Micrococcales	Micrococcaceae	*Glutamicibacter*	*sp.*	-	-	+
ASV40	Actinobacteria	Micrococcales	Microbacteriaceae	*Microbacterium*	*sp.*	-	-	+
ASV45	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas*	*sp.*	-	-	+
ASV46	Actinobacteria	Micrococcales	Brevibacteriaceae	*Brevibacterium*	*Brevibacterium*	-	-	+
ASV48	Bacilli	Bacillales	Planococcaceae	*Lysinibacillus*	*sp.*	-	-	+
ASV53	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	*sp.*	-	+	+
ASV56	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*brevis*	-	+	+
ASV68	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	*sp.*	-	-	+
ASV72	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Mesorhizobium*	*sp.*	-	-	+
ASV89	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	*sp.*	-	-	+
ASV91	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Corynebacterium*	*flavescens*	-	-	+
ASV93	Bacilli	Bacillales	Planococcaceae	*Lysinibacillus*	*sp.*	-	-	+
ASV94	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*sp.*	-	+	-
ASV105	Bacilli	Bacillales	Planococcaceae	*Lysinibacillus*	*sp.*	-	-	+
ASV108	Bacilli	Bacillales	Planococcaceae	*Lysinibacillus*	*sp.*	-	-	+
ASV110	Bacilli	Bacillales	Planococcaceae	*Lysinibacillus*	*sp.*	-	-	+
ASV114	Bacilli	Bacillales	Planococcaceae	*Lysinibacillus*	*sp.*	-	-	+
ASV115	Bacilli	Bacillales	Planococcaceae	*Lysinibacillus*	*sp.*	-	-	+
ASV117	Bacilli	Bacillales	Planococcaceae	*Lysinibacillus*	*sp.*	-	-	+
ASV152	Bacilli	o__	f__	*g__*	*sp.*	+	+	+
ASV189	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	*sp.*	-	-	+
ASV193	c__	o__	f__	*g__*	*sp.*	-	-	+
ASV383	Bacilli	Lactobacillales	Leuconostocaceae	*Leuconostoc*	*sp.*	-	+	-

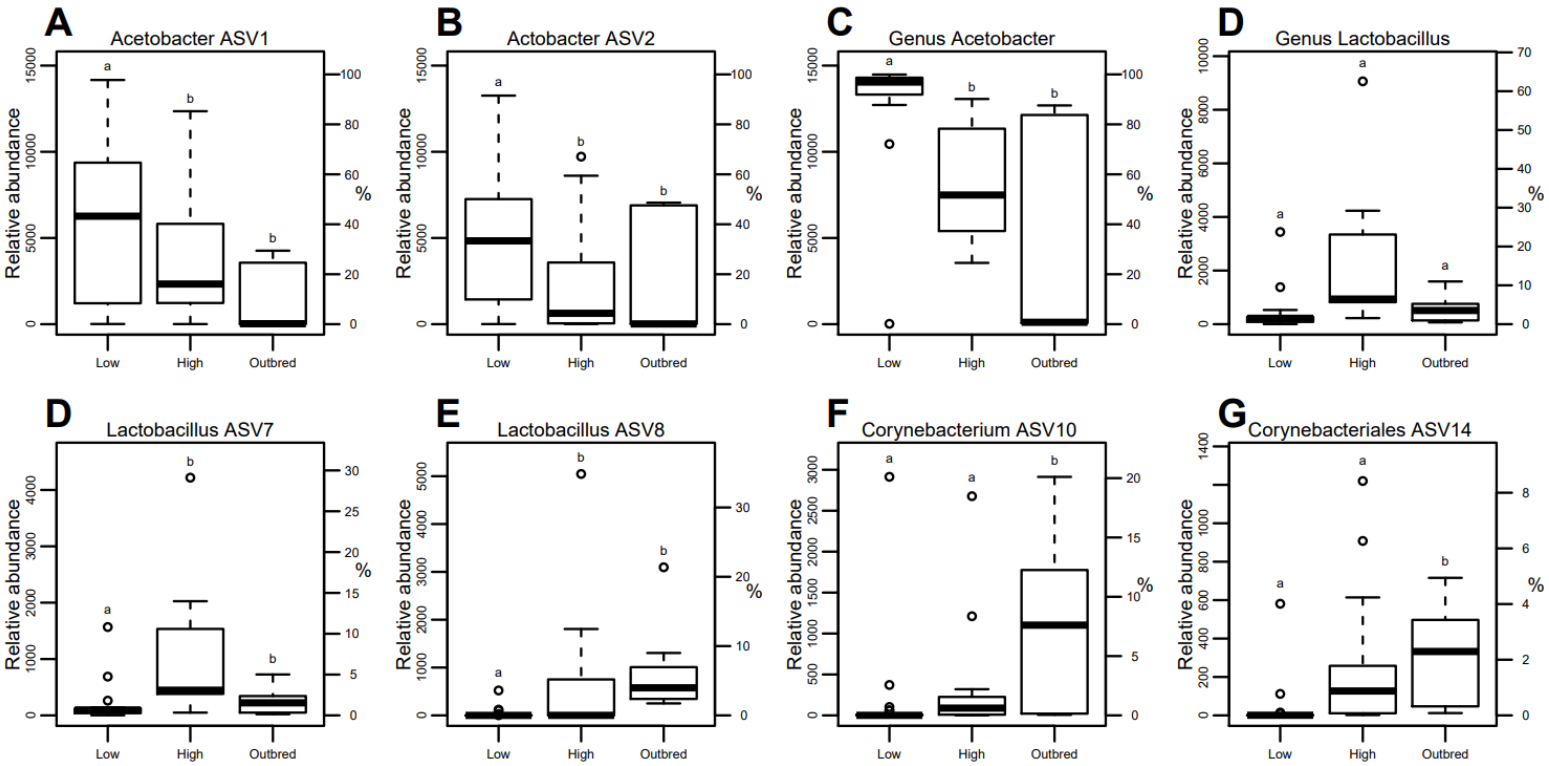


Figure 4.

Relative abundance of differentially enriched bacterial ASVs and genera **A-C.** *Acetobacter* ASVs and genus, **D-E.** *Lactobacillus* ASVs and genus, and **F-G.** Corynebacteriales ASVs in the three host genetic variations groups; low genetic variation (Low), high genetic variation (High), and outbred. The abundance axes are not scaled the same for all of the ASVs and genera because *Acetobacter* constitute the majority of the microbiome of the low genetic variation group. Letters denote significant differences in relative abundance of ASVs or genera between fly groups (DESeq2 Wald test adjusted $p < 0.05$). The percent reads are relative to the number to which all samples were initially rarefied (14,488 reads/sample).

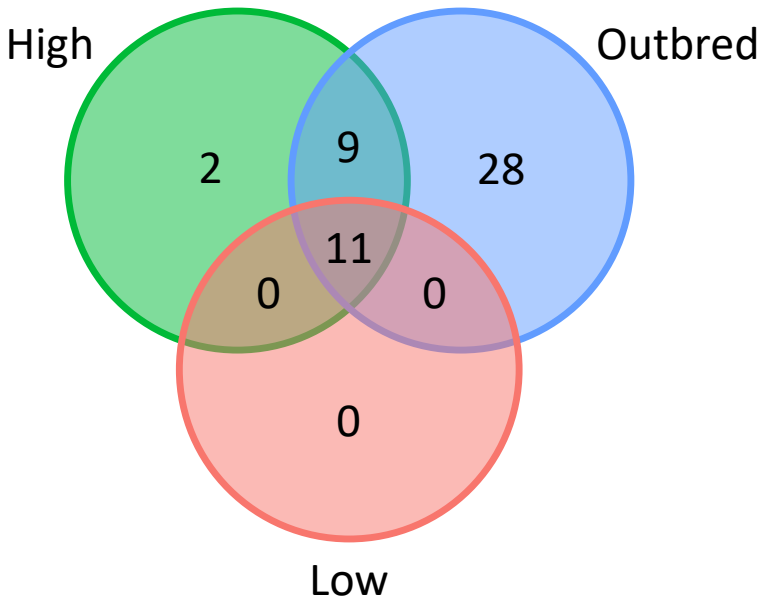


Figure 5.

Venn-diagram showing that the core microbiomes of the low genetic variation lines (Low; red) are subsets of those present in the higher genetic variation lines (High; green) and outbred lines (blue), with 11/11 low genetic variation group ASVs represented in the high genetic variation group, and 20/22 high genetic variation group ASVs represented in the outbred group. The taxonomy of individual ASVs can be seen in **Table 2**.

259 *Bacterial co-abundance networks decreased in complexity with lower host genetic variation*

260 In parallel to the DESeq analysis, which highlighted ASVs and genera, which were categorically
261 enriched in the different fly lines, co-abundance network analysis across the microbiome dataset was
262 performed to identify bacterial groups that are co-varying in relative abundance with the host fitness
263 traits of the lines and co-varying with each other (**Fig. 6**). Fly egg-to-adult viability and nucleotide
264 diversity co-varied with a large cluster of bacterial ASVs within the same modular cluster (**Fig. 6A**).
265 Generally, viability co-varied with a larger group of bacterial ASVs than nucleotide diversity, giving
266 40 and 13 associations, respectively, while 12 of these ASVs co-varied with both viability and
267 nucleotide diversity. Interestingly, most of the ASVs that co-varied with viability were either
268 differentially enriched in the outbred flies or in both the outbred and high genetic variation flies (**Fig.**
269 **6B**). The ASVs that were differentially enriched in both outbred and high genetic variation flies
270 belonged to 24 different taxonomic groups (**Fig. 6B, S7A**). In contrast, the two *Acetobacter* ASVs that
271 were significantly enriched in the low genetic variation flies belonged to separate modular clusters as
272 highly prevalent negatively correlated ASVs (**Supplementary Fig. S7A**). The smaller modules were
273 often exclusively made up of related ASVs, notably modules with *Lactobacillus* and *Enterococcus*
274 species. The networks of each individual fly group (**Fig. 6C,D,E**) revealed that inbreeding the flies
275 resulted in a lower number of co-varying ASVs, and decreased the degree of connectivity (i.e. the
276 number of significant correlations) and overall complexity of the network, with the latter consisting of
277 only a few ASVs in a modular structure. Having a higher host genetic variation did not result in a higher
278 degree of covariation among ASVs among inbred lines. Host genetic variation was both positively and
279 negatively correlated with the abundance of bacteria in the outbred network, while host genetic variation
280 did not significantly co-vary with bacterial abundance in the high and low genetic variation networks
281 (at correlation adjusted $p < 0.05$). Moreover, the ASVs belonging to the *Acetobacter*, that were
282 significantly enriched in the low genetic variation flies, displayed covariation only with *Lactobacillus*
283 among the inbred flies, (Fig. 6E), in contrast to those ASVs in the outbred flies.

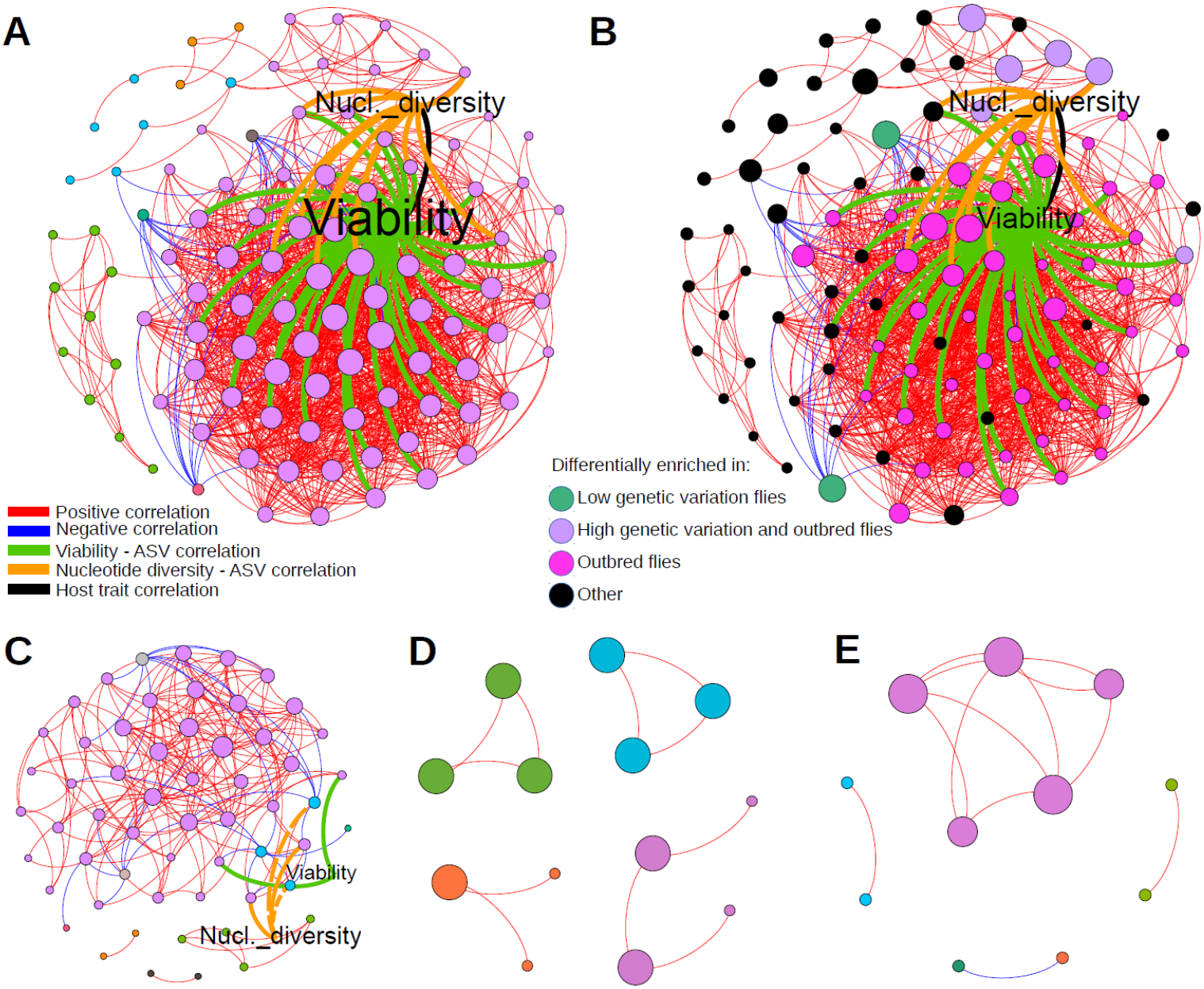


Figure 6.

Co-abundance networks of the fly microbiome. ASVs present in at least 30 reads in total and in at least three samples; correlations > 0.5 (or <-0.5) and fdr-corrected p-values < 0.05 are shown. The nodes are individual ASVs and the host fitness traits, egg-to-adult viability (Viability) and nucleotide diversity (Nucl. diversity), while the edges represent positive and negative correlations, and correlations linking host fitness traits and bacterial ASVs (which were positive correlations). The network containing samples from all of the fly groups (**A**), the outbred (**C**), high genetic variation (**D**), and low genetic variation (**E**) groups are shown with node sizes displaying the degree of connectivity (relative within each figure) and colors marking the modularity structure (i.e. in which community, or cluster, the ASVs belong to based on the leiden algorithm; **A, C, D, E**). The orange and green edges connecting Viability

294 and Nucleotide diversity were positive except for two instances in **C** where the negative correlations
295 are displayed with dotted lines. In **B**, node sizes display the number of samples within which the ASVs
296 are present, and colors mark the major groups in which the ASVs are differentially enriched. The
297 taxonomic assignment of each node can be seen in **Supplementary Fig. S7**.

298 # Discussion

299 In this study, we investigated the effects of host genetic variation on the microbiome diversity in *D.*
300 *melanogaster* lines with experimentally manipulated levels of genetic variation. Consistent with a
301 growing body of literature (Sommer & Bäckhed 2013; Ericsson & Franklin 2015; Mueller & Sachs
302 2015; Sampson & Mazmanian 2015; Sison-Mangus *et al.* 2015; Moghadam *et al.* 2018; Douglas 2019),
303 we show that the microbial community of the host is strongly linked to the fitness of the host, in our
304 case the viability of offspring. In addition, we link reductions in host genetic variation with microbiome
305 diversity and clearly show that microbiome diversity is lower in lines with reduced genetic variation,
306 regardless of whether we use richness or relative abundance metrics (**Fig. 2**). Only a few previous
307 studies have investigated the association between components of the microbiota and host genetics in
308 invertebrates, and those that did have mainly focused on genetic variability in the host's ability to control
309 the presence and/or abundance of specific endosymbionts (e.g., Mouton *et al.* 2007; von Burg *et al.*
310 2008; Chong & Moran 2016; Simhadri *et al.* 2017). Conversely, a few studies have investigated how
311 endosymbionts can affect genetic variation in the host by distorting sex-ratios, such as through
312 endosymbiont induction of cytoplasmic incompatibility or parthenogenesis (Engelstädter & Hurst
313 2009), potentially decreasing the effective population size and increasing genetic drift (Feldhaar 2011;
314 Ferrari & Vavre 2011) or by influencing population dynamics and dispersal possibly affecting host
315 genetic variation (Leonardo & Mondor 2006; Goodacre *et al.* 2009).

316 Despite recent calls for an integration of microbiome research in evolutionary and conservation
317 biology (Bahrndorff *et al.* 2016; Grueber *et al.* 2019; Hauffe & Barelli 2019; Trevelline *et al.* 2019;
318 West *et al.* 2019; Cullen *et al.* 2020; Moeller & Sanders 2020; Henry *et al.* 2021), little progress has
319 been made on experimentally testing fundamental associations between population size, host genetic

variation and microbial diversity. Here we provide a novel demonstration that restricting host population size has a profound impact on both the richness and the diversity of host-associated microbiomes, and that effects of host genetic variation and microbial richness interact synergistically in affecting host fitness. We also provide evidence that increased microbial diversity in the lines is associated with a stronger evolutionary response to stressful environments compared to responses seen in lines with less microbial diversity (**Supplementary Fig. S6**). Thus, we show that populations with high host genomic variation harbor the most diverse microbial community, and *vice versa* for populations with low genetic variation, and that these lines are least resilient to evolutionary pressure. According to the hologenome hypothesis, the holobiont constitutes a single unit of selection, thus our results suggest that small and fragmented populations have a reduced potential for responding to selection pressures not only due to reduced genetic variation and high rates of inbreeding but also due to reduced microbiome variation, effectively reducing hologenomic variation. Maintaining a diverse microbiome community may therefore be crucial for a host's responsiveness to environmental change (Zilber-Rosenberg & Rosenberg 2008; Feldhaar 2011; Bordenstein & Theis 2015; Henry *et al.* 2021).

Our study sheds light on the 'inheritance' of microbiomes and on hosts as 'environments' for the microbiota. While many animals, including a range of insects, have transovarial transmission of bacterial symbionts, i.e. via the egg to the embryo (McFall-Ngai *et al.* 2013; Robinson *et al.* 2019), the microbiome of captive *D. melanogaster*, especially the gut bacteria, are mainly horizontally acquired (Wong *et al.* 2016), and consist mostly of microbes from the diet and from microbes expelled into the immediate environment by conspecifics or predecessors (Blum *et al.* 2013). Interestingly, our results show that the 'high genetic variation' bottlenecked populations had a markedly reduced diversity of microbes, low numbers of co-varying bacteria, and lower fitness compared to the outbred control group, despite there being no difference in genetic variation between the two groups (see Ørsted *et al.* 2019 for an elaborated discussion of sampling of alleles during experimental bottlenecks). This could indicate that a reduction in host population size during a population bottleneck constrains the amount of microbial diversity available for random 'sampling' for the next generation, similar to the effects of genetic drift on host genetic variation. Indeed, the greater degree of variation in the microbiome

347 community membership, i.e. unweighted UniFrac-based ordination, among the inbred flies as compared
348 to the outbred group strongly suggests a community shift driven by repeated population bottlenecks
349 (**Fig. 3**). Similar to drift, these effects could be exacerbated in small populations, but unlike genetic
350 drift, which is a stochastic process, the effects of bottlenecks yield seemingly directional and predictable
351 effects on microbiome diversity.

352 The trend of decreasing microbiome diversity with increasing host inbreeding has also been found
353 in inbred populations of Diannan small-ear pigs and tortoises (Yuan *et al.* 2015; Wei *et al.* 2020). This
354 gives support to the notion that a decreased ability of inbred hosts to harbor diverse and stable
355 microbiomes could be a general pattern, although these studies did not link microbial diversity and
356 genetic variation to fitness effects or evolutionary responses. Previous experiments on *Drosophila* have
357 highlighted the impact of fly and larval density on microbial communities and how this can impact
358 fitness, producing a type of Allee effect (Wertheim *et al.* 2002). Simultaneously, a reduction in fitness
359 due to the genetic effects of population bottlenecks could in turn result in flies that cannot harbor some
360 components of the bacterial community, which could be especially critical if microbes with specific
361 metabolic functions are lost (West *et al.* 2019). We are unable to tease apart these effects in the current
362 study.

363 Despite this, we show that while host genetic variation has the strongest association with fitness, the
364 microbiome diversity also contributes, acting synergistically with host effects. We envision that
365 increased homozygosity within hosts in a population provides a less optimal habitat for maintaining
366 stable and diverse microbial communities. In this respect, a number of studies have previously reported
367 that hosts which are unhealthy and/or ill harbor different and less diverse microbiomes compared to
368 their healthier counterparts (Mistry *et al.* 2017; Gould *et al.* 2018; Fei *et al.* 2019; Saleem *et al.* 2019;
369 Wagener *et al.* 2020). Moreover, host genotypes have been found to profoundly affect the microbiome
370 composition, as well as their stability and beneficial effects on the host (Bonder *et al.* 2016; Rayes *et*
371 *al.* 2016; Adair *et al.* 2020), while other studies have shown that microbiomes affect host fitness,
372 development, and even allele frequency and host evolution (Goulet 2015; Wang *et al.* 2018; Rudman *et*
373 *al.* 2019). Given that flies from inbred lines with high nucleotide diversity in our study harbored less

374 diverse microbiomes and had lower fitness than flies from outbred lines which had similar nucleotide
375 diversity, we suggest that the microbiome is important in reducing fly fitness at high levels of genetic
376 variation typical in natural populations. On the other hand, lower nucleotide diversity correlated with
377 even lower microbial diversity and host fitness, suggesting that, when inbreeding results in low host
378 genetic variation, microbial diversity loss is primarily caused by reduced host fitness. Perhaps the less
379 optimal host 'habitat' for microbes in lines with low genetic diversity results in less favorable growth
380 conditions. It has been known for a long time that productivity of *Drosophila* can increase when there
381 is high genetic heterogeneity in cultures, presumably because multiple genotypes can better utilize
382 different aspects of the environment (Pérez-Tomé & Toro 1982; Hoffmann & Nielsen 1985). The
383 genetically diverse host populations could provide more variable environments for microbes, allowing
384 a greater diversity of microbes to persist, or support greater microbiome diversity through a higher
385 number of functionally important host genotype-microbial taxon-specific interactions. Tests of these
386 ideas in the context of small populations with restricted genetic variation require further studies that
387 might examine the impact of e.g. antibiotics on host and microbe associations with fitness. Other
388 possibilities for future research in this area involve the transfer of beneficial microbiomes to see if these
389 can "rescue" low fitness populations. Such experiments have been suggested in other contexts to
390 improve robustness and productivity of livestock and agricultural cultivars (Mueller & Sachs 2015). It
391 remains unexplored whether such fitness improvements by microbiome transfer can alleviate the
392 negative impacts commonly associated with low genetic variation like inbreeding depression.

393 The outbred lines investigated in our study have a much more complex network of co-varying, and
394 interacting microbiomes (**Fig. 6**), where removal of individual species is expected to have less impact
395 whereas in low fitness lines the removal of one key species may cause more dramatic effects. This
396 suggests that outbred lines harbour healthier and more robust microbial 'ecosystems' than bottlenecked
397 lines, where functional redundancy within the diverse microbial community of outbred flies promotes
398 community stability and subsequent host health. The importance of functional redundancy in host-
399 associated microbiome stability and host health has been previous demonstrated in various hosts as well
400 as many other ecosystems (Moya & Ferrer 2016; Liang *et al.* 2020). Notably, it is now widely

401 acknowledged that taxonomically diverse microbiomes harbor robust and stable functional redundancy,
402 where disturbance in the environment is countered, up to a point, by the resilience built upon functional
403 redundancy and high taxonomic diversity in a community. The global co-variation network in our flies
404 suggests that microbial diversity shifts gradually across a wide continuum of host genetic variation.
405 However, co-variation results from individual fly lines more clearly show that the tipping point between
406 community resilience and functional redundancy and communities suffering from more stochastic
407 microbe-microbe associations, and hence reduced functional redundancy, relates more to inbreeding
408 than genetic variation.

409 This bears some resemblance to ecological patterns observed in microbiomes of hosts living in
410 disturbed habitats (Amato *et al.* 2013; Barelli *et al.* 2015; Ingala *et al.* 2019). For instance, howler
411 monkeys inhabiting degraded, more homogenous forests had a less diverse gut microbiome compared
412 to conspecifics in non-fragmented forests, and as a result had lost the microbial metabolic pathway to
413 detoxify plant compounds in the leaves of their diet (Barelli *et al.* 2015). In the same way in which
414 diversity confers resilience in macro-ecological systems (Goodman 1975; Memmott *et al.* 2004), where
415 species-rich communities are less susceptible to invasion as more niches are occupied and limiting
416 resources are used more efficiently, such processes could be important for the robustness of microbial
417 ecosystems within hosts (Lozupone *et al.* 2012; Coyte *et al.* 2015). A high diversity of commensal
418 microbes could mean functional redundancy (Hauffe & Barelli 2019; West *et al.* 2019), thus an
419 increased resilience towards shifts in functional diversity, and better protection against pathogens
420 (Jaenike *et al.* 2010; Koskella & Bergelson 2020).

421 In conclusion, we demonstrate that restricting host population sizes is associated with a reduction in
422 diversity of host-associated microbiomes. We observe effects of both host genetic variation and
423 microbial diversity in explaining host fitness, however the patterns of causality remain unclear, i.e. we
424 suggest that it is not an all-or-nothing effect, but rather a continuum across nucleotide diversity where
425 fitness and microbiome depletion become relatively more important drivers. It is similarly difficult to
426 establish whether fitness differences are due to beneficial or potential pathogenic bacteria, partly
427 because pathogenicity depends on many factors including composition of the whole microbial

community present (Simhadri *et al.* 2017), and age of the host (Ayyaz & Jasper 2013). Despite this, we show clear effects of host population bottlenecks on the diversity of the microbiome, similar to effects normally observed in sampling of genetic alleles during genetic drift, and we show that both the reduction in microbiome diversity and genetic variation is tightly linked to host fitness and evolutionary potential. Therefore, microbial diversity in environmental samples could be a useful proxy for population fitness and potentially adaptability. Lastly, our results open up multiple avenues for further studies, such as transplantation of microbiomes as a means of 'rescuing' populations that suffer from inbreeding depression, potentially relevant to species of conservation concern.

Materials and methods

Fly population bottlenecks

The *D. melanogaster* flies used in the study originated from 232 wild females caught at Oakridge winery in the Yarra Valley, Victoria, Australia. These females contributed equally to a mass bred population of 1000 individuals. The flies were maintained on a 12:12 h light:dark cycle at 19°C on a standard laboratory food composed of yeast (60g/L water), sucrose (40g/L water), oatmeal (30g/L water), and agar (16g/L water) mixed with tap water. Nipagen (12mL/L water) and acetic acid (1.2mL/L water) was added to prevent fungal growth. From the mass bred population, we created 40 lines of each of three different expected levels of inbreeding for a total of 120 lines, from which we could obtain nucleotide diversity measures for 109 lines. This is described in detail in Ørsted *et al.* (2019). In summary, lines from the three levels of inbreeding experienced 2, 3, and 5 consecutive generations of bottlenecks each consisting of two males and two females (census size, N=4) revealing expected inbreeding coefficients of F=0.125, 0.219 and 0.381, respectively. After having reached the desired inbreeding level, we flushed the population sizes to 200 individuals. Simultaneously, we established 10 control lines that were assumed outbred as they were maintained at minimum 500 individuals.

452 *Fly lines selected for microbiome analysis*

453 We obtained a measure of the realised genetic variation in all inbred and outbred lines using genotyping-
454 by-sequencing (GBS) to calculate nucleotide diversity (π) from genomic SNPs (described in detail in
455 Ørsted *et al.* (2019); average number of SNPs ± sd = 26,877 ± 4,061). For the microbiome analysis, we
456 aimed at comparing two groups of fly lines: one group with low genetic variation and overall low
457 performance and one with high genetic variation and high overall performance. For a quantitative
458 selection of these groups, we calculated a composite measure of performance as the sum of standardised
459 values of viability and nucleotide diversity (Z-score). We selected the 25 lowest ranking lines and the
460 25 highest ranking lines based on this summed Z-score (50 lines total; **Supplementary Fig. S1**).
461 Because we selected lines regardless of their inbred/outbred status, nine of the ten outbred control lines
462 were included in the 'high genetic variation' lines due to their high Z-scores sum. In all analysis, we
463 differentiate between three groups of flies: 'low' and 'high' genetic variation lines within the inbred
464 lines and 'outbred' (OB) lines that have not been through population bottlenecks. Following establishing
465 the lines, population sizes were flushed to ca. 200 individuals per line, and in the F1 generation egg-to-
466 adult viability was assessed by distributing 15 eggs into each of five vials per line and calculating the
467 proportion of eggs developing successfully to the adult stage (Ørsted *et al.* 2019). Flies used for
468 microbiome analysis consisted of flies emerging from the egg-to-adult viability assessment (merged
469 across the 5 vials) that were snap frozen in liquid nitrogen at 2-3 days of age and subsequently stored at
470 -80°C.

471

472 *16S rRNA gene sample preparation and sequencing*

473 The whole fly genomic DNA was extracted following the same protocol as has been described in Ørsted
474 *et al.* (2019). In brief, 15 randomly collected male flies from each experimental line was homogenized
475 by bead beating at 2x6 s cycles and 6500 rpm using a Precellys mechanical homogenizer (Bertin
476 Techologies, Montigny le Bretonneux, France), and the DNA was purified with the DNeasy Blood &
477 Tissue kit (QIAGEN, Hilden, Germany) with modifications specific for extracting insect tissues. The
478 V1-V3 hypervariable regions of the bacterial 16S rRNA gene was amplified and multiplexed for

479 Illumina sequencing according to Albertsen *et al.* (2015), and the library pool was sequenced on a
480 MiSeq sequencer using the MiSeq Reagent kit v3, 2x300bp paired-end configuration, and 20% PhiX
481 control spike-in.
482
483 *Microbiome analysis*
484 The demultiplexed paired-end reads were quality filtered, assembled to make consensus amplicon reads,
485 clustered into ASVs, and taxonomically assigned using the in-house workflow AmpProc version
486 5.1.0.beta2.11.1 (https://github.com/eyashiro/AmpProc), which relies on USEARCH version
487 11.0.66_i86linux64 (Edgar 2010) sequenced reads processing and FastTree version 2.1.10 (Price *et al.*
488 2009) for tree building. The endosymbiont *Wolbachia* ASV was removed manually from the ASV table.
489 Next, QIIME version 1.9.1 (Caporaso *et al.* 2010) was used to rarefy all of the samples to the smallest
490 acceptable sample size of above 10,000 reads, i.e. 14,488 reads per sample (single_rarefaction.py), and
491 to generate the observed and estimated chao1 richness and Shannon-Wiener and Simpsons diversity
492 values for each sample (alpha_diversity.py), weighted and unweighted UniFrac matrices
493 (beta_diversity.py), and core microbiome groups (compute_core_microbiome.py). R version 3.5.0 was
494 used for downstream analysis and statistics. To assess the composition of core microbiomes, we first
495 normalized the dataset to eight samples per fly group (which is the number of outbred samples in the
496 study after filtering samples with low read counts) by randomly keeping three, two, and three samples
497 from the high, medium, and low inbreeding levels, respectively, from the high and low genetic variation
498 groups (Ørsted *et al.* 2019), and all of the outbred samples. Next, we calculated as belonging to the core
499 microbiome, the percentage to the nearest tenth if we were to accept a prevalence of an ASV in 80% of
500 the samples in each fly group.
501 Non-metric multidimensional plots based on the UniFrac matrices were generated with the R-
502 package 'vegan' (version 2.5-2 (Oksanen *et al.* 2018)). The effect of two host traits (nucleotide
503 diversity, egg-to-adult viability) (Ørsted *et al.* 2019) were calculated with the envfit function and the
504 significant envfit values ($p < 0.05$) of the fly host traits were displayed as arrows and fly host trait
505 gradients across the ordination space as isolines using the ordisurf function. PERMANOVA analysis

using vegan's adonis function was undertaken for the UniFrac matrices as a function of fly lines, nucleotide diversity and viability. Differentially enriched ASVs were identified with DESeq2 (version 1.22.2 (Love *et al.* 2014)) using the Wald test, contrasting across three levels (low and high genetic variation and outbred fly groups), and accepting the enriched ASVs with adjusted $p < 0.05$. The same approach was used on the genus-level frequency table. To test for differences in measures of microbiome diversity between the genetic variation groups, we performed Kruskal-Wallis tests and pairwise Wilcox's t-tests between groups.

Generalised linear mixed models

To assess drivers of fitness measured as egg-to-adult viability, we fitted generalised linear mixed effect models (GLMMs) in the R-package 'lme4' (Bates *et al.* 2015) assuming a binomial distribution with logit link function. We fitted viability as a function of nucleotide diversity and microbiome diversity (four different measures as described above) and their interaction as fixed effects; replicate vials were included as a random effect, as flies from the same vial are not independent. The full models were compared with individual univariate models of either nucleotide diversity or microbiome diversity by χ^2 difference tests. We detected no over-dispersion in any of the models (residuals to df ratio > 0.31; $\chi^2 > 76.02$; $p > 0.99$). Conditional coefficients of determination of the GLMMs interpreted as the variance explained by the entire model, including both fixed and random effects, were calculated as $R^2_{GLMM(c)} = \frac{\sigma_f^2+\sigma_\alpha^2}{\sigma_f^2+\sigma_\alpha^2+\sigma_\varepsilon^2}$, where σ_f^2, σ_α^2, σ_ε^2 are the variances of the fixed effect components, the random effects and the residual variance, respectively (see 'delta-method' in Nakagawa *et al.* (2017)).

Co-occurrence network analysis

For co-occurrence network analysis, the ASV table of all of the fly samples was used for the global network, and the subset of ASV tables generated for the core microbiomes analysis for outbred, high genetic variation, and low genetic variation groups were used as the starting frequency tables. In each of these ASV frequency tables, the fly nucleotide diversity and egg-to-adult viability measurements

were included. The ASVs present in fewer than three samples and ASVs with less than 30 reads in the respective frequency tables were removed. The 'Hmisc' R-package (Harrell 2021) was used to generate Spearman correlation matrices, and the upper triangle was used to generate the network edge list. Only edges with FDR-corrected $p < 0.05$ and correlation coefficients > 0.5 (or < -0.5) were kept. In Gephi version 0.9.2, the network was generated with the Fruchterman Reingold algorithm and minor crossing of edges were corrected manually to improve the visualization of the clusters. From among Gephi's built-in features, the leiden algorithm was used to calculate the modularity structure using edge weights and default settings, and the degree of connectivity was calculated as the number of nodes with which each node is connected by an edge.

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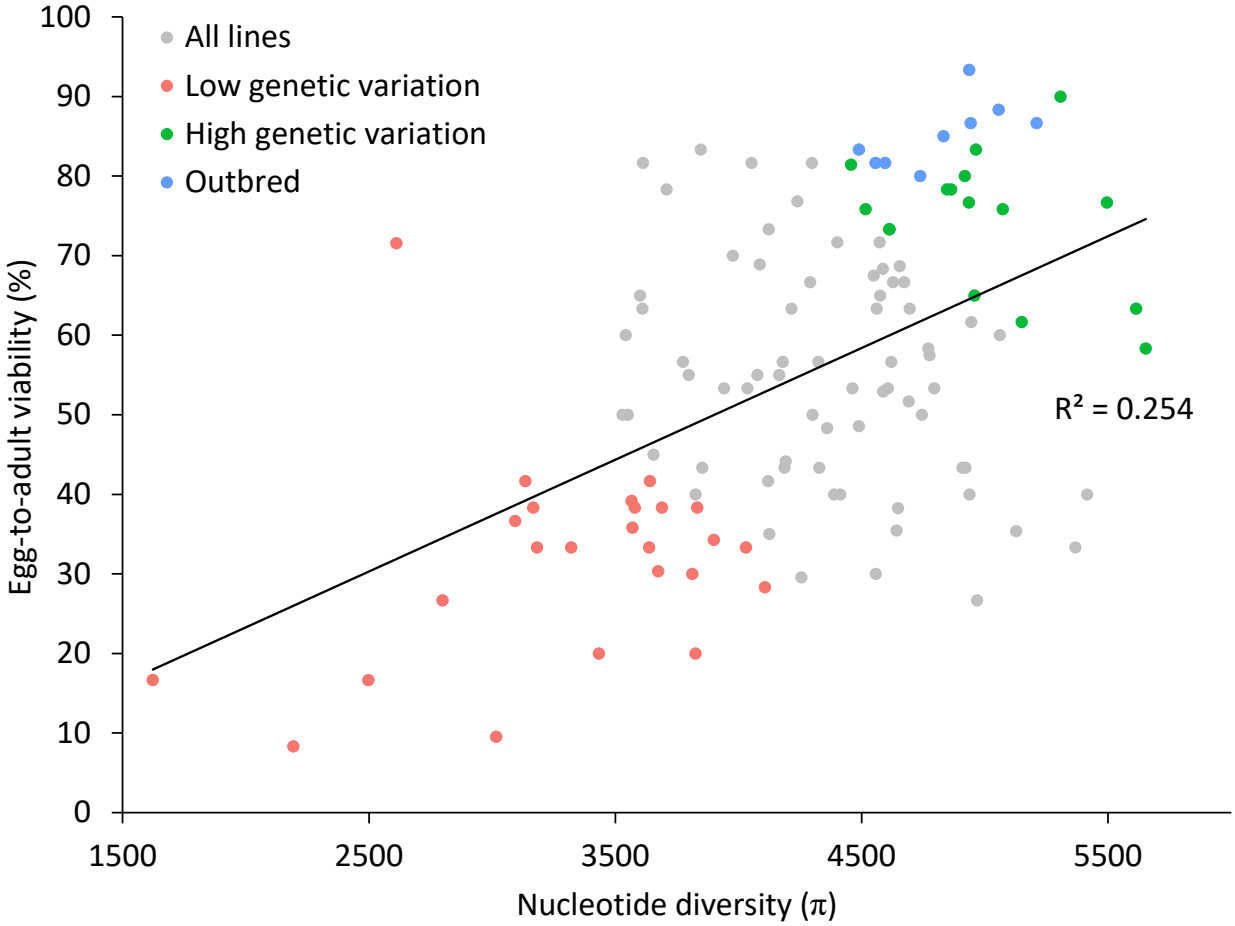
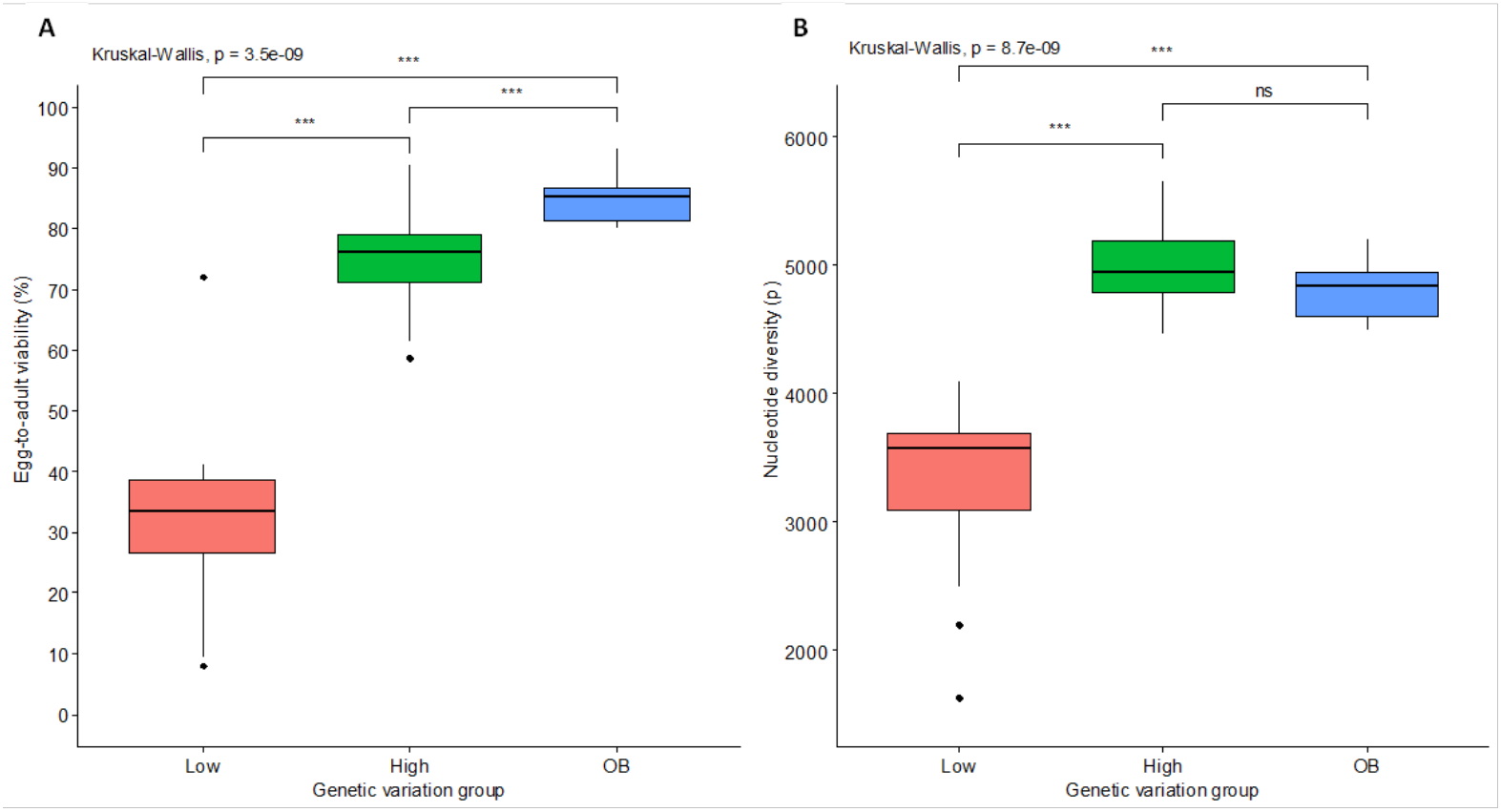
748

Supplementary Information



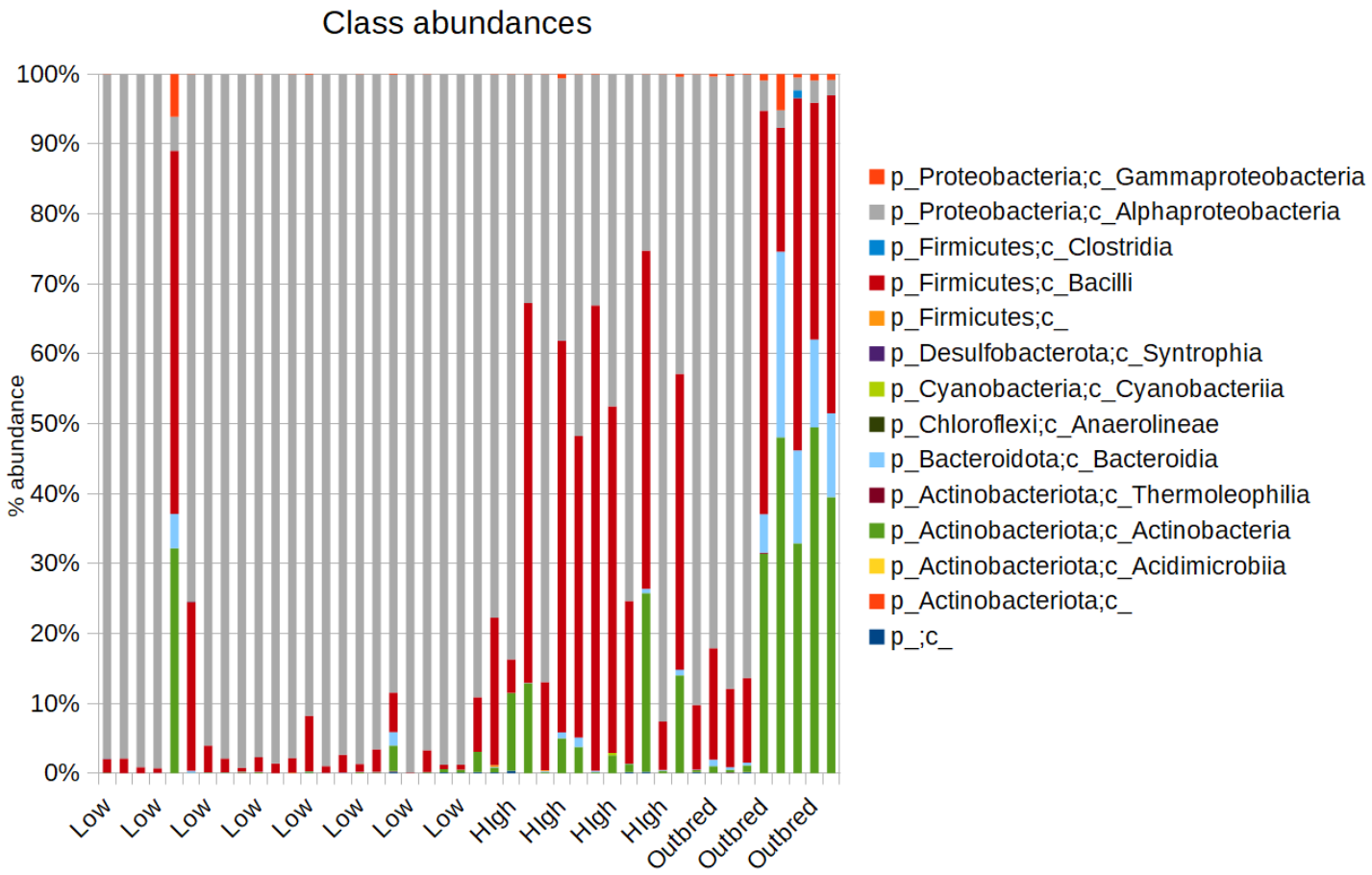
Supplementary Fig. S1.

Scatterplot of line mean egg-to-adult viability (%) and nucleotide diversity (π) as obtained through GBS of 119 lines (grey circles; from Ørsted et al. 2019). From these lines, we selected three groups of lines for analysis of the microbiome, 25 lines with low overall performance (red circles), and 25 lines with high performance (green and blue). This selection was based on a composite measure of the overall performance calculated as the sum of standardized viability and nucleotide diversity. Because we selected lines regardless of their inbred/outbred status, the 'high' group included nine of the outbred controls as they generally had very high fitness and genetic variation. To distinguish between the effects of genetic variation within inbred lines and population bottlenecks, we therefore differentiate between three genetic variation categories: 'Low genetic variation' and 'High genetic variation' lines and 'Outbred' (OB) control lines. The solid line represent the linear regression on all lines with the R^2 value shown.



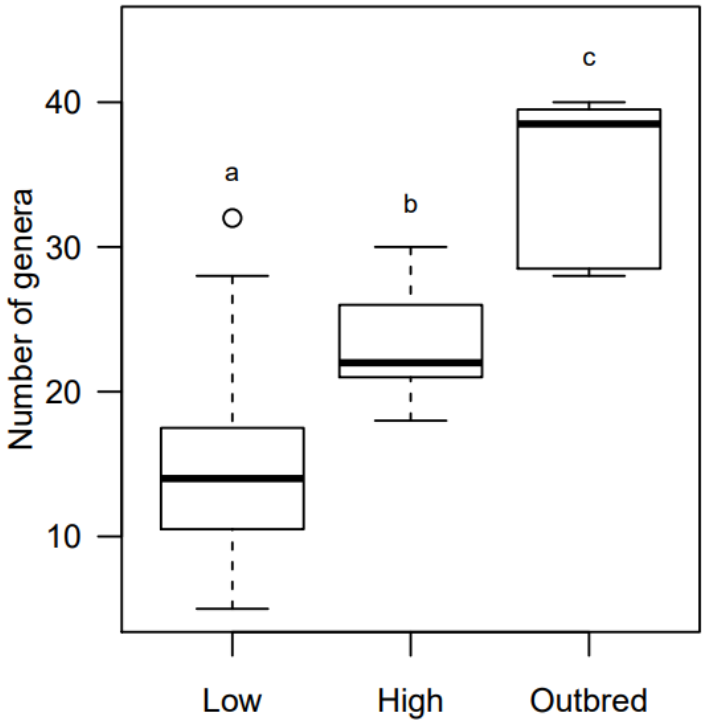
Supplementary Fig. S2.

Both the high genetic variation ('High') and the outbred ('OB') lines exhibited a significantly higher viability (**A**) and nucleotide diversity (**B**) than the low genetic variation lines ('Low'), and viability of OB lines was higher than that of the high genetic variation lines, while nucleotide diversity was indistinguishable between these two groups (two-sample Wilcox's t-tests; $p < 0.05$). The p values of a Kruskal-Wallis test show significant effects of group, while asterisks denote the results of pairwise Wilcoxon's t-tests between groups; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; and ns: $p > 0.05$.



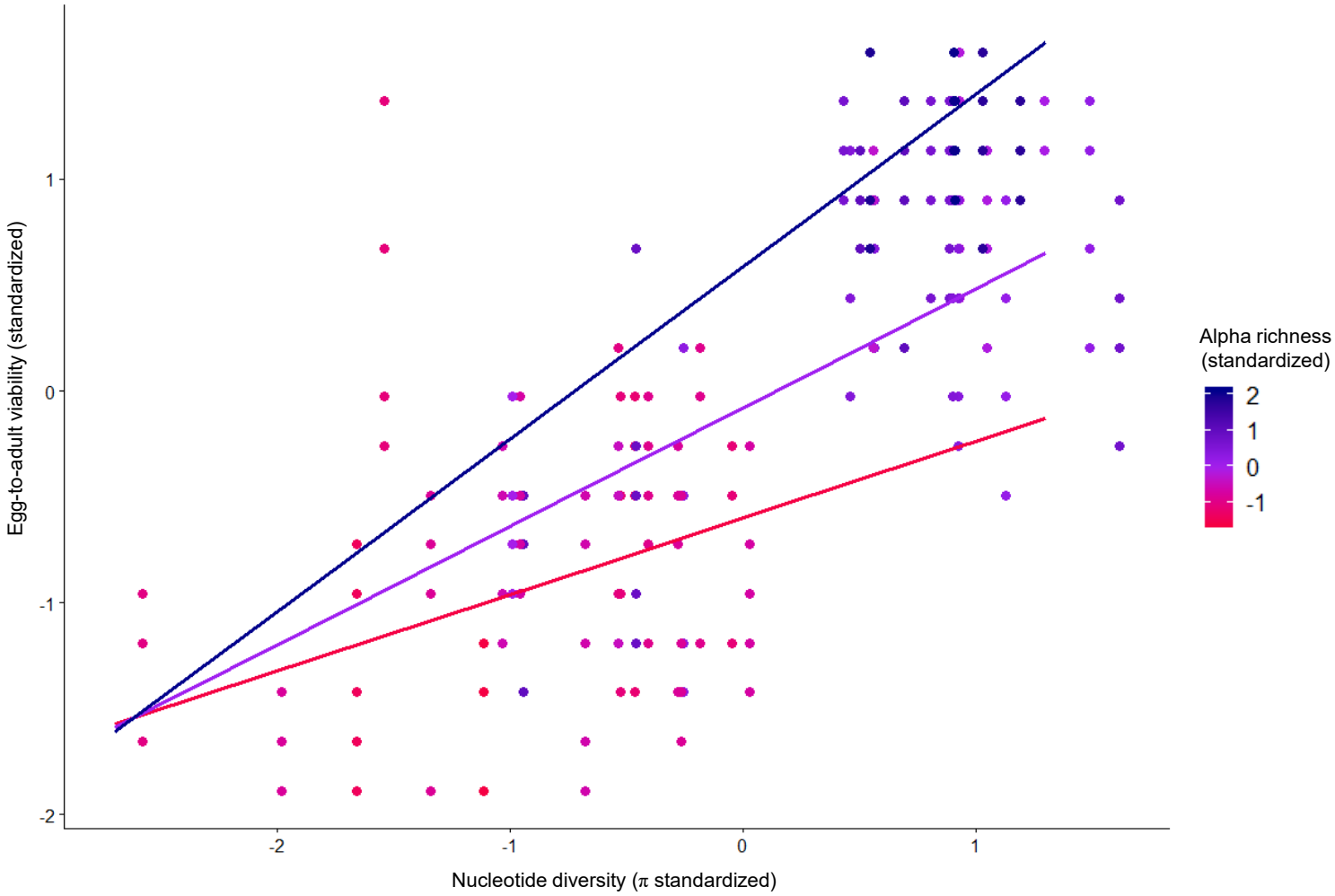
Supplementary Fig. S3.

Bar chart of class-level taxonomic composition of the fly microbiomes in the three major groups of host genetic variation (Low: low genetic variation, High: high genetic variation, and Outbred: outbred flies).



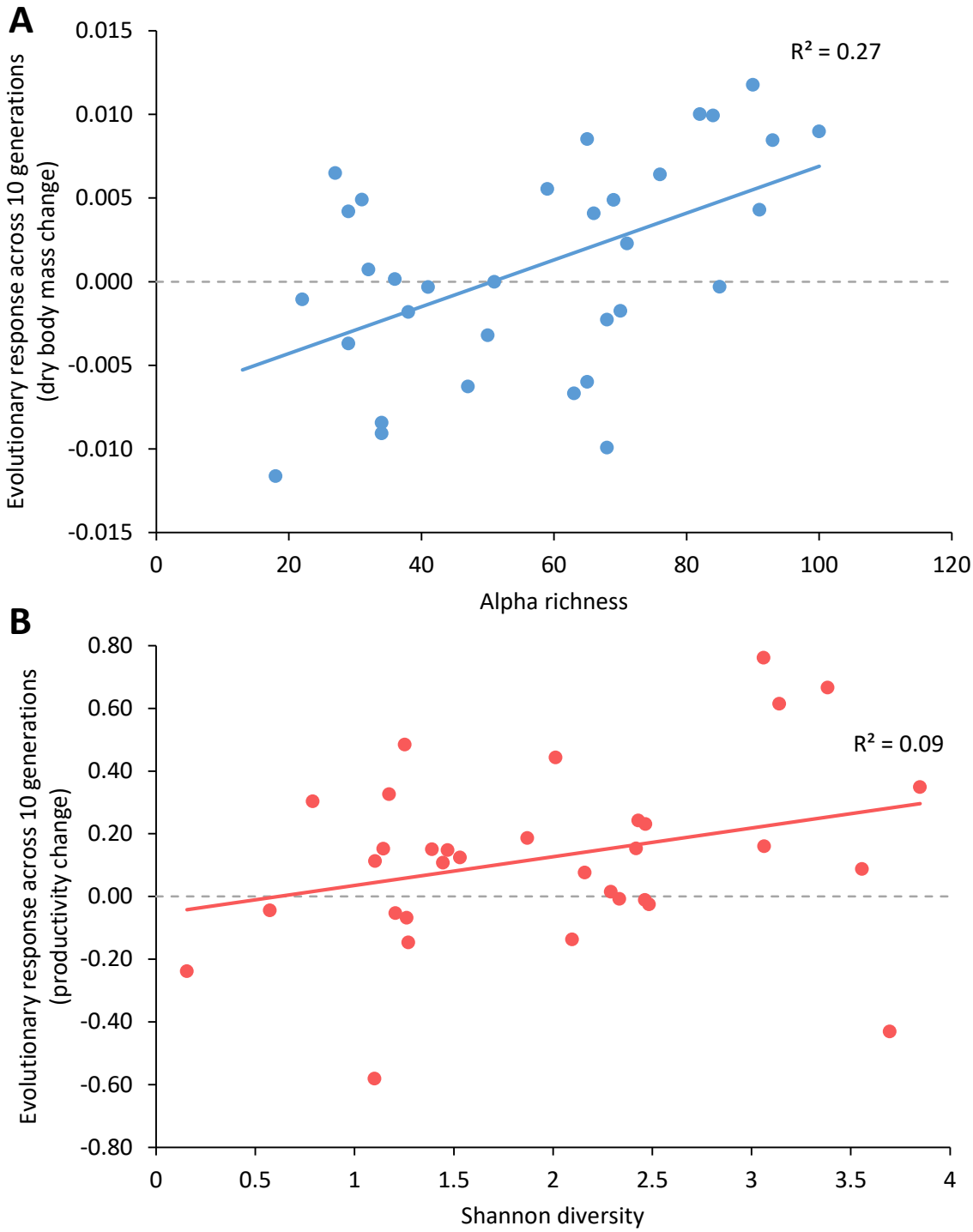
Supplementary Fig. S4.

Box plot of the number of bacterial genera harboured in each line, grouped according to fitness (Low: low genetic variation, High: high genetic variation, and Outbred: outbred flies). Letters denote significant differences in mean number of genera (Tukey's HSD test).



Supplementary Fig. S5

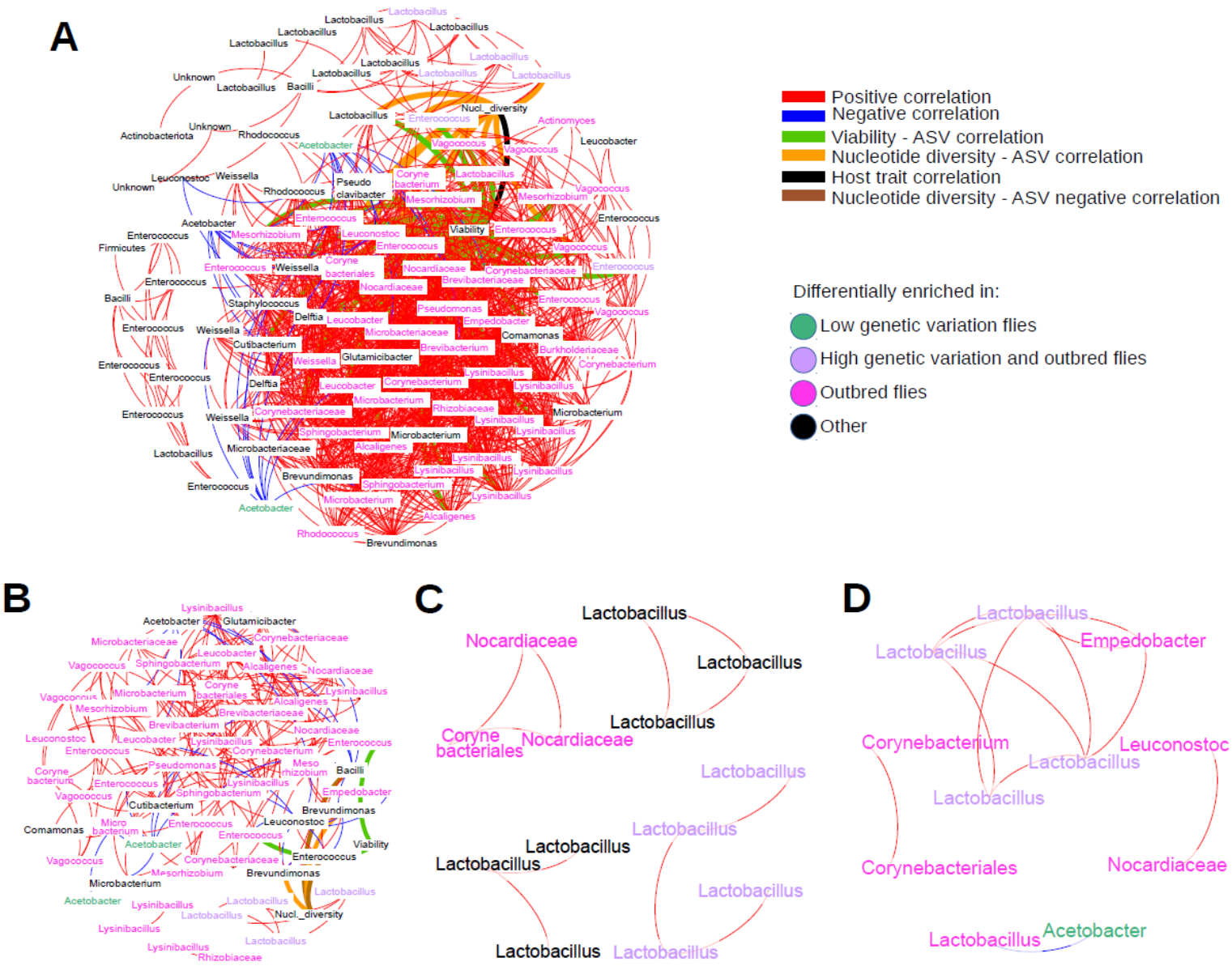
Scatterplot of egg-to-adult viability (z-standardised) from 5 vials per line as a function of nucleotide diversity (π z-standardised) for each of the 50 lines investigated for microbial diversity; the colour of points denote observed ASV richness (alpha richness, z-standardised). The three regression lines represent regressions on lines with the highest (blue), mean (purple), and lowest (red) alpha richness, visualizing the significant positive interaction between nucleotide diversity and microbiome richness in explaining fitness of the host population (see **Table 1** for GLMM results).



Supplementary Fig. S6

Using data from Ørsted *et al.* (2019) from the set of lines in the present study, we associate microbial diversity with evolutionary responses in two traits: **A.** dry body mass, and **B.** productivity. Here evolutionary responses are defined as the slope of an ordinary linear regression across 10 generations of rearing on a stressful medium. We correlated all four measures of microbial diversity used in the present study to these two traits (eight comparisons), but here we only included the cases where there was a significant effect of microbial diversity. For both traits, we found an effect of microbial diversity

(alpha richness regressed on response in dry body mass: $t_{29} = 2.09$, $p = 0.045$, and Simpson's diversity regressed on response in productivity: $t_{29} = 2.42$, $p = 0.022$), but no effect of genetic variation, π ($t_{29} = -1.07$, $p = 0.92$, and $t_{29} = -0.14$, $p = 0.89$, for dry body mass and productivity, respectively). For details on assessment of these phenotypes, see Ørsted *et al.* (2019).



Supplementary Fig. S7

Co-abundance networks of the fly microbiome (closely resembles **Fig. 6** in main text, except here we supply the ASVs for each node). ASVs present in at least 30 reads in total and in at least three samples, and correlations of > 0.5 (or <-0.5) and fdr-corrected *p*-values of < 0.05 are shown. The nodes are individual ASVs and the host fitness traits, egg-to-adult viability (Viability) and nucleotide diversity (Nucl. diversity), while the edges represent positive and negative correlations, and correlations linking host fitness traits and bacterial ASVs (which were positive correlations). The network containing samples from all of the fly groups (**A**), the outbred (**B**), high genetic variation (**C**), and low genetic variation (**D**) groups are shown with the taxonomic assignment of each node. Non-highlighted nodes that could not be taxonomically assigned are left as ASV identifier numbers. The highlighted nodes are color-coded according to the major DESeq differential abundance groups. The

807 results of both the leiden and degree were plotted into the network graphs as node colors and node
808 sizes, respectively. For cross-referencing the DESeq2 differentially enriched ASVs in the networks,
809 only the most robust differential abundance patterns were displayed, notably the ASVs that were
810 systematically enriched in the low genetically diverse flies in both the low-high and low-outbred
811 pairwise comparisons (low), the ASVs systematically enriched in the outbred flies (outbred), and
812 those depleted in the low diverse flies (high & outbred).

Supplementary Table S1

Results of general linear mixed models (GLMMs) of egg-to-adult viability as a function of nucleotide diversity (NuclDiv) and microbiome diversity and their interaction as fixed effects. The measures of microbiome diversity are divided into alpha richness indices; estimated ASV richness (chao1), and diversity indices accounting for relative abundances as well (Shannon-Wiener index (Shannon) and Simpson's index (Simpson) in each of their own model. Both dependent and independent variables are scaled (Z-standardization) to allow direct comparison of effect sizes. Replicate vial ID were included as a random effect, as flies from the same vial are not considered independent. Conditional coefficients of determination of the GLMMs $\left(R^2_{\text{full model}}\right)$ interpreted as the variance explained by the entire model, including both fixed and random effects, is shown. Asterisks denote the significance of individual variables or interactions; *** $p < 0.001$; ** $p < 0.01$; and * $p < 0.05$. The full model including both dependent variables and their interaction is compared with individual models with either nucleotide diversity or alpha diversity with a χ^2 test.

Microbiome diversity measure	Fixed effects	Estimate	Std. Error	z value	p	
chao1 estimated ASV Richness (chao1)	Intercept	0.134	0.081	1.658	0.0972	
$R^2_{full\ model}$ = 0.353	NuclDiv	0.767	0.084	9.173	4.61E-20	***
	chao1	0.490	0.083	5.882	4.04E-09	***
	NuclDiv*chao1	0.141	0.086	1.632	0.1026	
	Random effects	**Std.Dev**				
	Vial	0.707				
	Comparison with individual models	**χ^2 df**	**χ^2**	**p**		
	NuclDiv; chao1; Full model	2	75.434	2.00E-16	***	
	Fixed effects	**Estimate**	**Std. Error**	**z value**	**p**	
Shannon-Wiener Index (Shannon)	Intercept	0.148	0.085	1.736	0.0826	
$R^2_{full\ model}$ = 0.354	NuclDiv	0.817	0.097	8.411	4.07E-17	***
	Shannon	0.411	0.093	4.430	9.41E-06	***
	NuclDiv*Shannon	0.105	0.088	1.193	0.2329	
	Random effects	**Std.Dev**				
	Vial	0.735				
	Comparison with individual models	**χ^2 df**	**χ^2**	**p**		
	NuclDiv; Shannon; Full model	2	67.413	2.30E-15	***	
	Fixed effects	**Estimate**	**Std. Error**	**z value**	**p**	
Simpson's Index (Simpson)	Intercept	0.130	0.078	1.660	0.0970	
$R^2_{full\ model}$ = 0.354	NuclDiv	0.896	0.090	9.997	1.57E-23	***
	Simpson	0.341	0.085	4.023	5.74E-05	***
	NuclDiv*Simpson	0.137	0.073	1.879	0.0603	
	Random effects	**Std.Dev**				
	Vial	0.750957				
	Comparison with individual models	**χ^2 df**	**χ^2**	**p**		
	NuclDiv; Simpson; Full model	2	86.078	2.00E-16	***	

827 **Supplementary Table S2**

828 DESeq2 table is found online as a Microsoft Excel spreadsheet. The log fold change calculations are
829 done on the ASV (L7 tabs in the spreadsheet) and genus level frequency tables (L6 tabs in the
830 spreadsheet). The DESeq contrasts were done among outbred, high and low genetic variation fly groups,
831 and the pairwise contrasts are displayed in separate sheets. For the most significant pairwise
832 comparisons ($p < 0.1$), the fly group in which the ASVs or genera are enriched is indicated.