

1 Key features of the genetic architecture and
2 evolution of host-microbe interactions revealed
3 by high-resolution genetic mapping of the
4 mucosa-associated gut microbiome in hybrid
5 mice

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21 **Abstract:** Determining the forces that shape diversity in host-associated bacterial
22 communities is critical to understanding the evolution and maintenance of
23 metaorganisms. To gain deeper understanding of the role of host genetics in shaping gut
24 microbial traits, we employed a powerful genetic mapping approach using inbred lines
25 derived from the hybrid zone of two incipient house mouse species. Further, we
26 uniquely performed our analysis on microbial traits measured at the gut mucosal
27 interface, which is in more direct contact with host cells and the immune system. A high
28 number of mucosa-associated bacterial taxa have significant heritability estimates;
29 heritabilities are greater for 16S rRNA transcript- compared to gene copy-based traits,
30 and interestingly, are positively correlated with cospeciation rate estimates. Genome-
31 wide association mapping identifies 443 loci influencing 123 taxa, with narrow genomic
32 intervals pinpointing promising candidate genes and pathways. Importantly, we
33 identified an enrichment of candidate genes associated with several human diseases,
34 including inflammatory bowel disease, and functional categories including innate
35 immunity and G-protein-coupled receptors. These results highlight key features of the
36 genetic architecture of mammalian host-microbe interactions and how they diverge as
37 new species form.

38

39 **Keywords:** microbiome; GWAS; cospeciation; codiversification; hybridization;
40 phylosymbiosis

41 Introduction

42 The recent widespread recognition of the gut microbiome's importance to host
43 health and fitness represents a critical advancement of biomedicine. Host phenotypes
44 affected by the gut microbiome are documented in humans (Ley et al., 2006; Turnbaugh
45 et al., 2009; Lynch and Pedersen, 2016), laboratory animals (Backhed et al., 2004;
46 Turnbaugh et al., 2008; Rolig et al., 2015; Rosshart et al., 2017; Gould et al., 2018), and
47 wild populations (Suzuki, 2017; Roth et al., 2019; Suzuki et al., 2020; Hua et al., 2020),
48 and include critical traits such as aiding digestion and energy uptake (Rowland et al.,
49 2018), and the development and regulation of the immune system (Davenport, 2020).

50 Despite the importance of gut microbiome, community composition varies
51 significantly among host species, populations, and individuals (Benson et al., 2010;
52 Yatsunenکو et al., 2012; Brooks et al., 2016; Rehman et al., 2016; Amato et al., 2019).
53 While a portion of this variation is expected to be selectively neutral, alterations of the
54 gut microbiome are on the one hand linked to numerous human diseases (Carding et al.,
55 2015; Lynch and Pedersen, 2016) such as diabetes (Qin et al., 2012), inflammatory bowel
56 disease (IBD) (Ott et al., 2004; Gevers et al., 2014) and mental disorders (Clapp et al.,
57 2017). On the other hand, there is evidence that the gut microbiome can play an
58 important role in adaptation on both recent- (Hehemann et al., 2010; Suzuki and Ley,
59 2020) and ancient evolutionary timescales (). Collectively, these phenomena suggest that
60 it would be evolutionarily advantageous for hosts to influence their microbiome.

61 An intriguing observation made in comparative microbiome research in the last
62 decade is that the pattern of diversification among gut microbiomes appears to mirror
63 host phylogeny (Ochman et al., 2010). This phenomenon, coined "phylosymbiosis"
64 (Brucker and Bordenstein, 2012a; Brucker and Bordenstein, 2012b; Lim and Bordenstein,
65 2020), is documented in a number of diverse host taxa (Brooks et al., 2016) and also
66 extends to the level of the phageome (Gogarten et al., 2021). Several non-mutually
67 exclusive hypotheses are proposed to explain phylosymbiosis (Moran and Sloan, 2015).
68 However, it is likely that vertical inheritance is important for at least a subset of taxa, as
69 signatures of co-speciation/-diversification are present among numerous mammalian
70 associated gut microbes (Moeller et al., 2016; Groussin et al., 2017; Moeller et al., 2019),
71 which could also set the stage for potential coevolutionary processes. Importantly,
72 experiments involving interspecific fecal microbiota transplants indeed provide
73 evidence of host adaptation to their conspecific microbial communities (Brooks et al.,
74 2016; Moeller et al., 2019). Further, cospeciating taxa were observed to be significantly
75 enriched among the bacterial species depleted in early onset IBD, an immune-related
76 disorder, suggesting a greater evolved dependency on such taxa (Papa et al., 2012;
77 Groussin et al., 2017). However, the nature of genetic changes involving host-microbe
78 interactions that take place as new host species diverge remains under-explored.

79 House mice are an excellent model system for evolutionary microbiome research, as
80 studies of both natural populations and laboratory experiments are possible (Suzuki,

81 2017; Suzuki et al., 2019). In particular, the house mouse species complex is comprised of
82 subspecies that hybridize in nature, enabling the potential early stages of
83 codiversification to be studied. We previously analyzed the gut microbiome across the
84 central European hybrid zone of *Mus musculus musculus* and *M. m. domesticus* (Wang et
85 al., 2015), which share a common ancestor ~ 0.5 million years ago (Geraldès et al., 2008).
86 Importantly, transgressive phenotypes (i.e. exceeding or falling short of parental values)
87 among gut microbial traits as well as increased intestinal histopathology scores were
88 common in hybrids, suggesting that the genetic basis of host control over microbes has
89 diverged (Wang et al., 2015). The same study performed an F₂ cross between wild-
90 derived inbred strains of *M. m. domesticus* and *M. m. musculus* and identified 14
91 quantitative trait loci (QTL) influencing 29 microbial traits. However, like classical
92 laboratory mice, these strains had a history of rederivation and reconstitution of their
93 gut microbiome, thus leading to deviations from the native microbial populations found
94 in nature (Rosshart et al., 2017; Org and Lusi, 2018), and the genomic intervals were too
95 large to identify individual genes.

96 In this study, we employed a powerful genetic mapping approach using inbred
97 lines directly derived from the *M. m. musculus* - *M. m. domesticus* hybrid zone, and
98 further focus on the mucosa-associated microbiota due to its more direct interaction
99 with host cells (Fukata and Arditi, 2013; Chu and Mazmanian, 2013), distinct functions
100 compared to the luminal microbiota (Wang et al., 2010; Vaga et al., 2020), and greater
101 dependence on host genetics (Spor et al., 2011; Linnenbrink et al., 2013). Previous
102 mapping studies using hybrids raised in a laboratory environment showed that high
103 mapping resolution is possible due to the hundreds of generations of natural admixture
104 between parental genomes in the hybrid zone (Turner and Harr, 2014; Pallares et al.,
105 2014; Škrabar et al., 2018). Accordingly, we here identify 443 loci contributing to
106 variation in 123 taxa, whose narrow genomic intervals (median <2Mb) enable many
107 individual candidate genes and pathways to be pinpointed. We identify a high
108 proportion of bacterial taxa with significant heritability estimates, and find that bacterial
109 phenotyping based on 16S rRNA transcript compared to gene copy-based profiling
110 yields an even higher proportion. Further, these heritability estimates also significantly
111 positively correlate with cospeciation rate estimates, suggesting a more extensive host
112 genetic architecture for cospeciating taxa. Finally, we identify numerous enriched
113 functional pathways, whose role in host-microbe interactions may be particularly
114 important as new species form.

115 Results

116 *Microbial community composition*

117 To obtain microbial traits for genetic mapping in the G2 mapping population, we
118 sequenced the 16S rRNA gene from caecal mucosa samples of 320 hybrid male mice
119 based on DNA and RNA (cDNA), which reflect bacterial cell number and activity,
120 respectively. After applying quality filtering and subsampling 10,000 reads per sample,
121 we identified a total of 4684 amplicon sequence variants (ASVs). For further analyses,
122 we established a "core microbiome" (defined in Methods), such that analyses were
123 limited to those taxa common and abundant enough to reveal potential genetic signal.
124 The core microbiome is composed of four phyla, five classes, five orders, eleven families,
125 27 genera, and 90 ASVs for RNA, and four phyla, five classes, six orders, twelve families,
126 28 genera and 46 ASVs for DNA. A combined total of 98 unique ASVs belong to the core,
127 of which 38 were shared between DNA and RNA (Suppl. Fig. 1). The most abundant
128 genus in our core microbiome is *Helicobacter* (Suppl. Fig. 2), consistent with a previous
129 study of the wild hybrid *M. m. musculus*/*M. m. domesticus* mucosa-associated
130 microbiome (Wang et al., 2015).

131 *Correlation between host genetic relatedness and microbiome structure*

132 To gain a broad sense of the contribution of genetic factors to the variability of
133 microbial phenotypes in our mapping population, we compared the kinship matrix
134 based on genotypes to an equivalent based on gut microbial composition, whereby ASV
135 abundances were used as equivalents of gene dosage. We found a significant correlation
136 between these matrices ($P = .001$; Suppl. Fig. 3), indicating a host genetic effect on the
137 diversity of the gut microbiota.

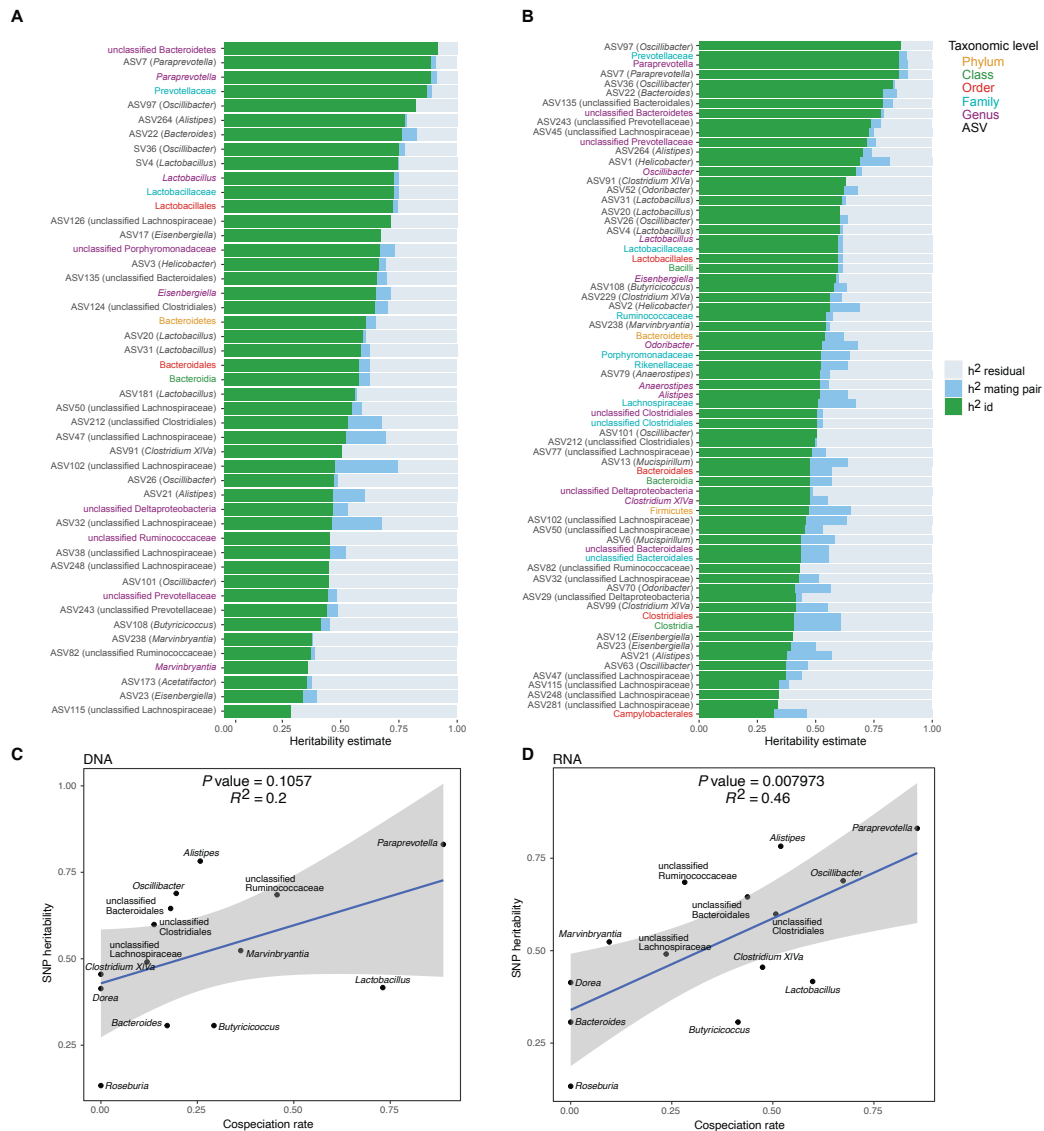
138 *SNP-based heritability*

139 Next, we used a SNP-based approach to estimate the proportion of variance
140 explained (PVE) of the relative abundance of taxa, also called the narrow-sense
141 heritability (h^2) or SNP-based heritability. Out of the 153 total core taxa, we identified 46
142 taxa for DNA and 69 taxa for RNA with significant heritability estimates ($P_{RLRT} < .05$),
143 with estimates ranging between 29 and 91% (see Fig. 1A-B and Suppl. Table 1). An
144 unclassified genus belonging to the phylum Bacteroidetes followed by ASV7 (genus
145 *Paraprevotella*), *Paraprevotella* and Paraprevotellaceae showed the highest heritability
146 among DNA-based traits (91.8%, 88.8%, 88.8%, and 87.1%, respectively; Fig. 1A), while
147 ASV97 (genus *Oscillibacter*), followed by Prevotellaceae, *Paraprevotella* and ASV7
148 (*Paraprevotella*) had the highest heritability among RNA-based traits (86.6%, 85.7%,
149 85.7%, and 85.6%, resp.; Fig. 1B). The heritability estimates for DNA- and RNA-based
150 measurements of the same taxa are significantly correlated ($P = 5.013 \times 10^{-8}$, $R^2=0.58$,
151 Suppl. Fig. 4), and neither measure appears to be systematically more heritable than

152 another, i.e. some taxa display higher RNA-based heritability estimates and others
 153 higher DNA-based estimates.

154 *Heritability estimates are correlated with predicted co-speciation rates*

155 In an important meta-analysis of the gut microbiome across diverse mammalian
 156 taxa, Groussin et al. (2017) estimated co-speciation rates of individual bacterial taxa by
 157 measuring the congruence of host and bacteria phylogenetic trees relative to the number
 158 of host-swap events. We reasoned that taxa with higher co-speciation rates might also
 159 demonstrate higher heritability, as these more intimate evolutionary relationships would
 160 provide a greater opportunity for genetic aspects to evolve. Intriguingly, we observe a
 161 significant positive correlation for RNA-based traits ($P = .008$, $R^2 = .46$, Fig. 1D) and a
 162 similar trend for DNA ($P = 0.1$; Fig. 1C). These results support the notion that
 163 cospeciating taxa evolved a greater dependency on host genes, and further suggest that
 164 bacterial activity may better reflect the underlying biological interactions.

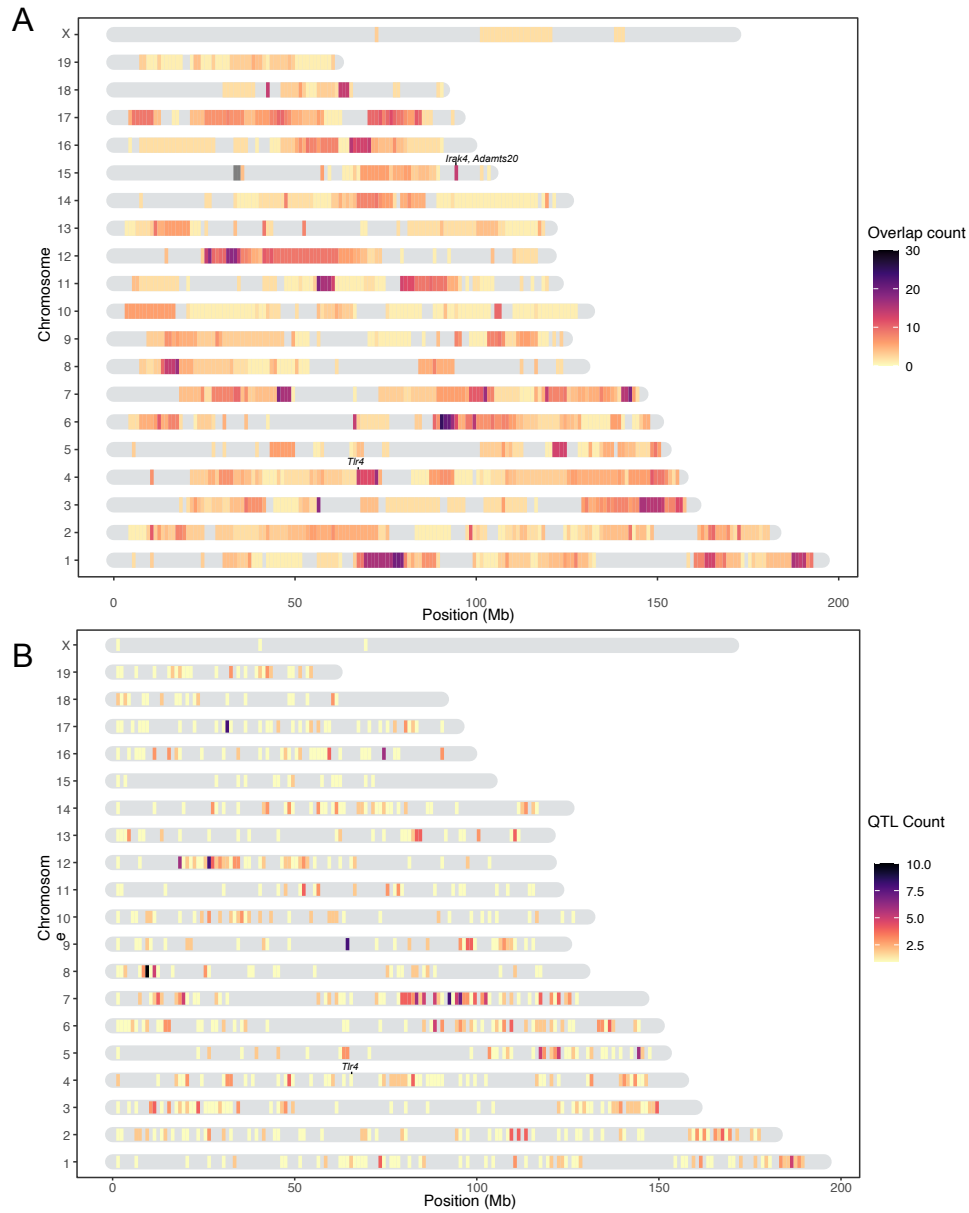


165 **Figure 1:** (A-B) Heritability estimates for the relative abundance of bacterial taxa. Proportion
166 of variance explained for each taxon on DNA level (A), and RNA level (B) for all SNPs (GRM) in
167 green, mating pair identifier in blue and residual variance in grey. Only significant heritability
168 estimates are shown ($P < .05$). The text labels on the y-axis are colored according to taxonomic
169 level: ASV in black, genus in purple, family in light blue, order in red, class in green, and phylum
170 in yellow. (C-D) Relationship between the heritability estimates for the relative abundance of
171 bacterial taxa and co-speciation rate for the same genus calculated by Groussin et al. (2017). DNA
172 level (C), and RNA level (D). The blue line represents a linear regression fit to the data and the
173 grey area the corresponding confidence interval.

174 *Genetic mapping of host loci determining microbiome composition*

175 Next, we performed genome-wide association mapping of the relative abundances
176 of core taxa, in addition to two alpha-diversity measures (Shannon and Chao1 indices),
177 based on 32,625 SNPs. We included both additive and dominance terms in the model to
178 enable the identification of under- and over-dominance (see Methods). While we found
179 no significant associations for alpha diversity at either the DNA or RNA level ($P > 1.53 \times$
180 10^{-6}), a total of 1099 genome-wide significant associations were identified for individual
181 taxa ($P < 1.53 \times 10^{-6}$, Suppl. Table 2), of which 443 achieved study-wide significance (P
182 $< 1.29 \times 10^{-8}$). Apart from the X chromosome, all autosomal chromosomes contained
183 study-wide significant associations (Fig. 2). Out of the 153 mapped taxa, 123 had at least
184 one significant association (Table 1). For the remainder of our analyses, we focus on the
185 results using the more stringent study-wide threshold, and combined significant SNPs
186 within 10 Mb into significant regions (Suppl. Table 3). The median size of significant
187 regions is 1.91 Mb, which harbor a median of 14 protein-coding genes. On average, we
188 observe 10 significant mouse genomic regions per bacterial taxon.

189 Of the significant loci with estimated interval sizes, we find 73 intervals (16.5%) that
190 are smaller than one Mb (Suppl. Table 4). The smallest interval is only 231 bases and
191 associated with the RNA-based abundance of an unclassified genus belonging to
192 Deltaproteobacteria. It is situated in an intron of the C3 gene, a complement component
193 playing a central role in the activation of the complement system, which modulates
194 inflammation and contributes to antimicrobial activity (Ricklin et al., 2016).



195 Figure 2: Heatmap of significant host loci from association mapping of bacterial
196 abundances. Karotype plot showing the number of significant loci found using a study-
197 wide threshold, where (A) plots the significance intervals, and (B) the significant SNP
198 markers on the chromosomes.

199 Table. 1. Overview of mapping statistics.

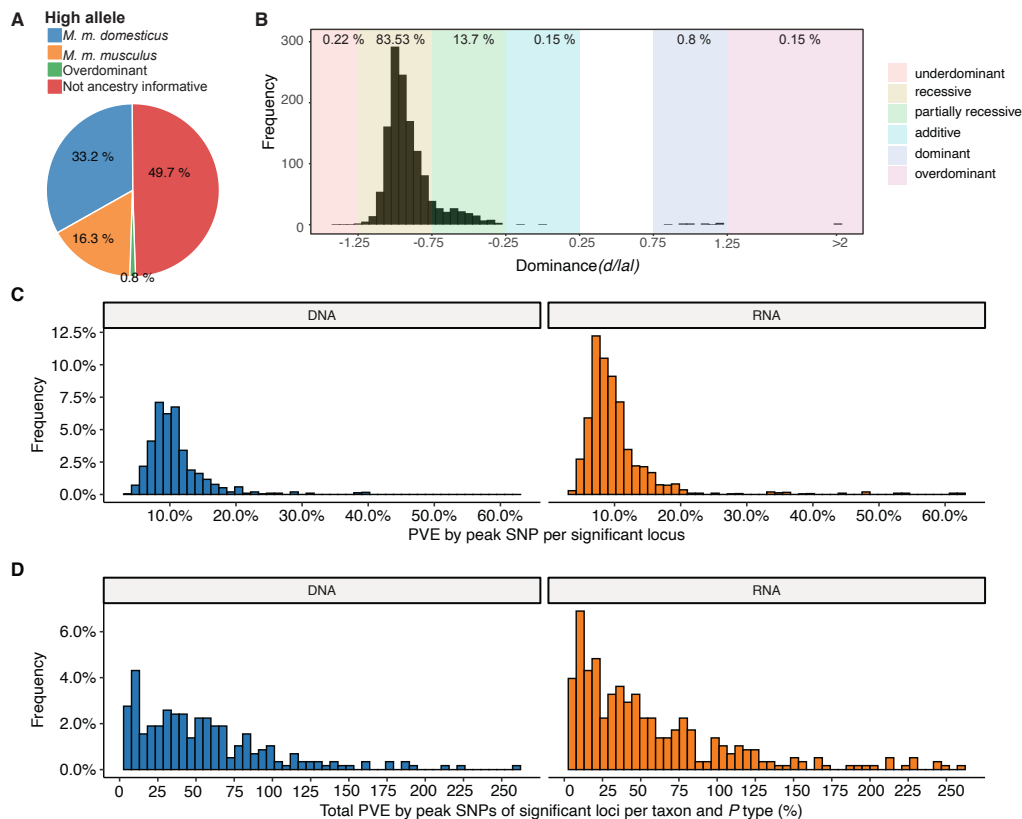
	DNA	RNA	Total	
200				
201	Mapped taxa	101	142	153
202	Taxa with significant loci	67	96	123
203	Median interval size (Mb)	1.52	2.29	1.91
204	Total significant loci	478	791	1269
205	Unique significant loci	179	313	443
206	Significant loci total P	91	167	233
207	Significant loci additive P	155	260	377
208	Significant loci dominance P	95	166	231
209	Median significant loci per trait	5	6	8
210	Median unique significant loci per trait	3	3	4
211	Median unique significant SNPs per locus	2	2.5	2
212	Median number of genes per locus	31	52	43
213	Median protein coding genes per locus	11	15	14

214 The significant genomic regions and SNPs are displayed in Figure 2A and 2B,
215 respectively. Individual SNPs were associated with up to 12 taxa, and significant
216 intervals with up to 30 taxa. The SNPs with the lowest P values were associated with the
217 genus *Dorea* and two ASVs belonging to *Dorea* (ASV184 and ASV293; Suppl. Fig. 5). At
218 the RNA level this involves two loci: mm10-chr4: 67.07 Mb, where the peak SNP is 13 kb
219 downstream of the closest gene *Tlr4* (UNC7414459, $P=2.31 \times 10^{-69}$, additive $P= 4.48 \times$
220 10^{-118} , dominance $P= 1.37 \times 10^{-111}$), and mm10-chr15: 94.4 Mb, where the peak SNP is
221 found within the *Adamts20* gene (UNC26145702, $P=4.51 \times 10^{-65}$, additive $P= 1.87 \times 10^{-113}$,
222 dominance $P= 1.56 \times 10^{-105}$; Fig. 2; Suppl. Fig. 5). Interestingly, the *Irak4* gene, whose
223 protein product is rapidly recruited after TLR4 activation, is also located 181 kb
224 upstream of *Adamts20*. The five taxa displaying the most associations were ASV19
225 (*Bacteroides*), *Dorea*, ASV36 (*Oscillibacter*), ASV35 (*Bacteroides*), and ASV98 (unclassified
226 Lachnospiraceae) (Suppl. Fig. 6).

227 *Ancestry, dominance, and effect sizes*

228 A total of 435 significant SNPs were ancestry informative between *M. m. musculus*
229 and *M. m. domesticus* (*i.e.* represent fixed differences between subspecies). To gain further
230 insight on the genetic architecture of microbial trait abundances, we estimated the
231 degree of dominance at each significant locus using the
232 d/a ratio (Falconer, 1996), where alleles with strictly recessive, additive, and dominant
233 effects have d/a values of -1, 0, and 1, respectively. As half of the SNPs were not ancestry
234 informative (Fig. 3A), it was not possible to consistently have a associated with one
235 parent/subspecies, hence we report $d/|a|$ such that it can be interpreted with respect to
236 bacterial abundance. For the vast majority of loci (83.53%), the allele associated with
237 lower abundance is dominant or partially dominant ($-1.25 < d/|a| < -0.75$; Fig. 3B). On
238 the basis of the arbitrary cutoffs we used to classify dominance, only a small proportion

239 of alleles are underdominant (0.22%; $d/|a| < -1.25$) or overdominant (0.15%; $d/|a| >$
 240 1.25). However for one-third of the significant SNPs, the heterozygotes display
 241 transgressive phenotypes, i.e. mean abundances that are either significantly lower (31%
 242 of SNPs)- or higher (2% of SNPs) than those of both homozygous genotypes.
 243 Interestingly, the *domesticus* allele was associated with higher bacterial abundance in
 244 two-thirds of this subset (33.2% vs 16.3% *musculus* allele; Fig. 3A).



245 **Figure 3:** Genetic architecture of significant loci. A) Source of the allele with the highest phenotypic value. B) Histogram of dominance values d/a of significant loci reveals a majority of loci acting recessive or partially recessive. C) Histogram showing the percentage of variance explained (PVE) by the peak SNP for DNA (blue, left) and RNA (orange, right). D) Collective PVE by lead SNPs of significant loci within a taxon. Values are calculated separately for each *P* value type (total, additive, and dominance).

251 Next, we estimated phenotypic effect sizes by calculating the percentage variance
 252 explained (PVE) by the peak SNP of each significant region. Peak SNPs explain between
 253 3% and 64% of the variance in bacterial abundance, with a median effect size of 9.3%
 254 (Fig. 3C). The combined effects of all significant loci for each taxon ranged from 4.9% to
 255 259%, with a median of 41.8% (Fig. 3D). Note, combined effects for many taxa (33 out of
 256 59) exceed SNP-heritability estimates (Fig. 1). While exceeding 100% explained variance
 257 is biologically possible, as loci can have opposite phenotypic effects, many of these are
 258 likely inflated due to the Beavis effect (Beavis, 1994).

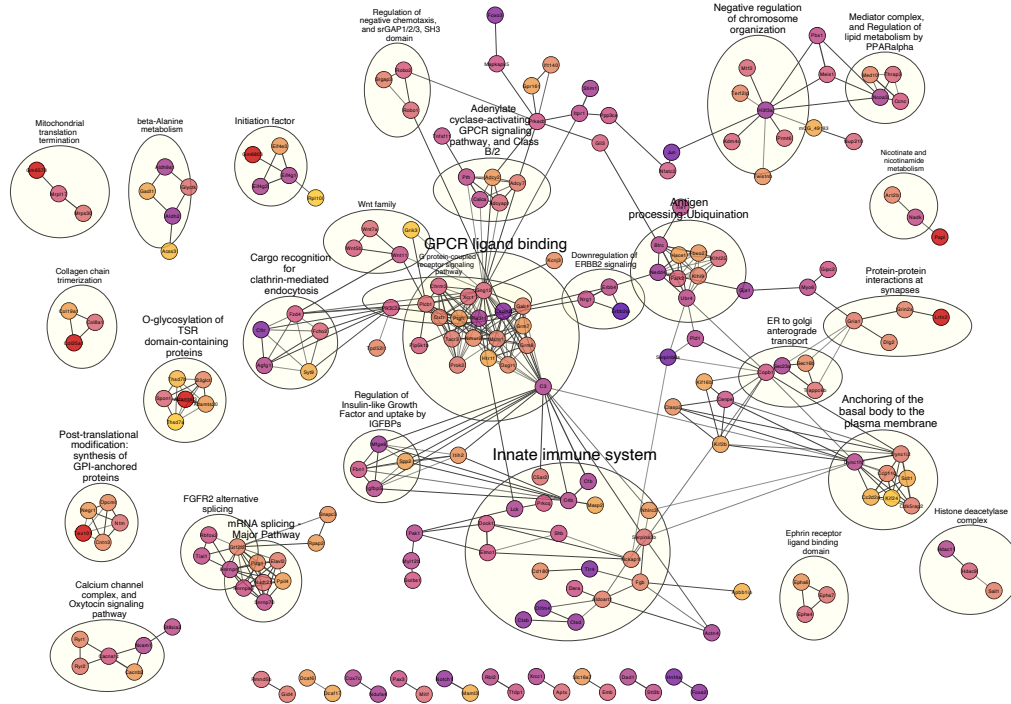
259 *Functional annotation of candidate genes*

260 In order to reveal potential higher level biological phenomena among the identified
261 loci, we performed pathway analysis to identify interactions and functional categories
262 enriched among the genes in significant intervals. We used STRING (Szklarczyk et al.,
263 2019) to calculate a protein-protein interaction (PPI) network of 925 protein-coding
264 genes nearest to significant SNPs (upstream and/or downstream). A total of 768 genes
265 were represented in the STRING database, and the maximal network is highly
266 significant (PPI enrichment P value: 2.15×10^{-14}) displaying 668 nodes connected by 1797
267 edges and an average node degree of 4.68. After retaining only the edges with the
268 highest confidence (interaction score > 0.9), this results in one large network with 233
269 nodes, 692 edges and ten smaller networks (Fig. 4).

270 Next, we functionally annotated clusters using STRING's functional enrichment
271 plugin. The genes of the largest cluster are part of the G protein-coupled receptor
272 (GPCR) ligand binding pathway. GPCRs are the largest receptor superfamily and also
273 the largest class of drug targets (Sriram and Insel, 2018). We then calculated the top ten
274 hub proteins from the network based on Maximal Clique Centrality (MCC) algorithm
275 with CytoHubba to predict important nodes that can function as 'master switches'
276 (Suppl. Fig. 7). The top ten proteins contributing to the PPI network were GNG12,
277 MCHR1, NMUR2, PROK2, OXTR, XCR1, TACR3, CHRM3, PTGFR, and C3, which are
278 all involved in the GPCR signaling pathway.

279 Further, we performed enrichment analysis on the 925 genes nearest to significant
280 SNPs using the *clusterprofiler* R package. We found 14 KEGG pathways to be over-
281 represented: circadian entrainment, oxytocin signaling pathway, axon guidance, calcium
282 signaling, cAMP signaling, cortisol synthesis and secretion, cushing syndrome, gastric
283 acid secretion, glutamatergic synapse, mucin type O-glycan biosynthesis, inflammatory
284 mediator regulation of TRP channels, PD-L1 expression and the PD-1 checkpoint
285 pathway in cancer, tight junction, and the *Wnt* signaling pathway (Suppl. Table 5, Suppl.
286 Fig. 8-9). Finally, genes involved in five human diseases are enriched, among them
287 mental disorders (Suppl. Fig. 10).

288 Finally, due to the observation of a significant enrichment of cospeciating taxa
289 among the bacterial species depleted in early onset IBD (Groussin et al., 2017) and the
290 evidence that IBD is especially associated with a dysbiosis in mucosa-associated
291 communities (Yang et al., 2020a; Daniel et al., 2021), we specifically examined possible
292 over-representation of genes involved in IBD (Khan et al., 2021) among the 925 genes
293 neighboring significant SNPs. We found 14 out of the 289 IBD genes, which was
294 significantly more than expected by chance (10 000 times permuted mean: 2.7, simulated
295 $P = .0001$; Suppl. Table 6). Interestingly, SNPs in five out of the 14 genes are associated
296 with ASVs belonging to the genus *Oscillibacter*, a cospeciating taxon known to decrease
297 during the active state of IBD (Metwaly et al., 2020).



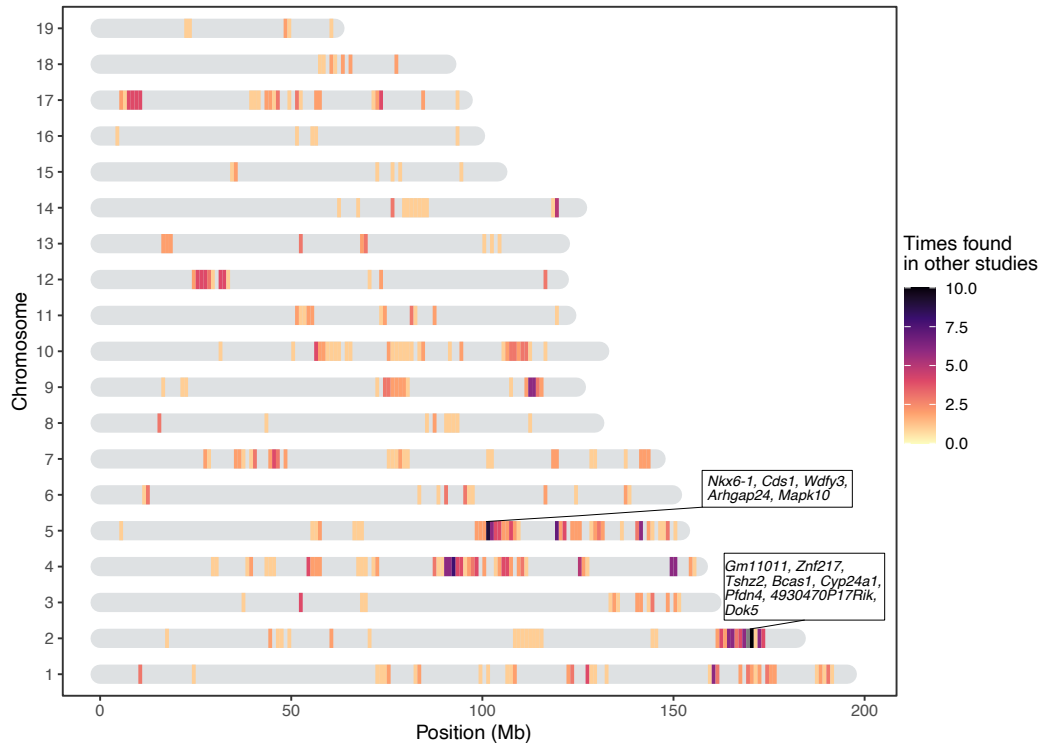
298 Figure 4: High confidence protein-protein interaction network of genes closest to SNPs
299 significantly associated with bacterial abundances. Network clusters are annotated using
300 STRING's functional enrichment (Doncheva et al., 2019). Nodes represent proteins and
301 edges their respective interactions. Only edges with an interaction score higher than 0.9
302 are retained. The width of the edge line expresses the interaction score calculated by
303 STRING. The color of the nodes describe the expression of the protein in the intestine
304 where yellow is not expressed and purple is highly expressed.

305 *Comparison of significant loci to published gut microbiome mapping studies*

306 Next, we compiled a list of 648 unique confidence intervals of significant
307 associations with gut bacterial taxa from seven previous mouse QTL studies (Benson et
308 al., 2010; McKnite et al., 2012; Leamy et al., 2014; Wang et al., 2015; Org et al., 2015;
309 Snijders et al., 2016; Kemis et al., 2019) and compared this list to our significance
310 intervals for bacterial taxa at both the DNA and RNA level (346 unique intervals).
311 Regions larger than 10Mb were removed from all studies. We found 434 overlapping
312 intervals, which is significantly more than expected by chance (10 000 times permuted
313 mean: 368, simulated $P=0.0073$, see Methods). Several of our smaller significant loci
314 overlapped with larger loci from previous studies and removing this redundancy left
315 186 significant loci with a median interval size of 0.78 Mb (Fig. 5). The most frequently
316 identified locus is located on chromosome 2 169-171 Mb where protein coding genes
317 *Gm11011*, *Znf217*, *Tshz2*, *Bcas1*, *Cyp24a1*, *Pfdn4*, *4930470P17Rik*, and *Dok5* are situated.

318 Additionally, we collected genes within genome-wide significant regions reported
319 in seven human microbiome GWAS (mGWAS) (Bonder et al., 2016; Turpin et al., 2016;

320 Goodrich et al., 2016; Wang et al., 2016; Hughes et al., 2020; Rühlemann et al., 2021;
321 Kurilshikov et al., 2021). However, no significant over-representation of genes was
322 found within our significance intervals ($P = .156$), nor within our list of genes closest to a
323 significant SNP ($P = .62$).



324 **Figure 5:** Heatmap showing the significant loci in this study that were previously
325 found in other QTL studies of the mouse gut microbiome. The genes present in
326 two repeatedly identified regions are depicted in boxes.

327 *Proteins differentially expressed in germ-free vs conventional mice*

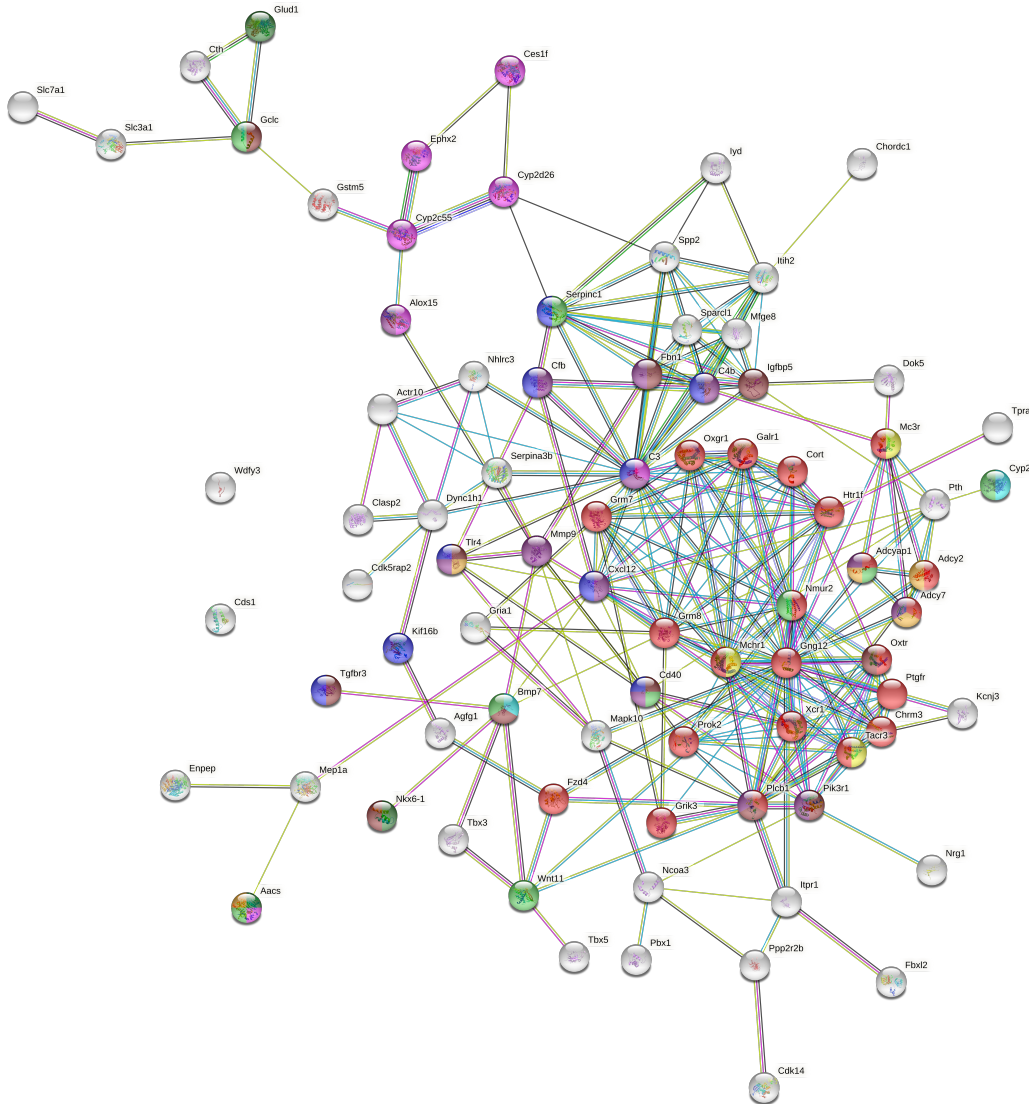
328 To further validate our results, we compared the list of genes contained within
329 intervals of our study to a list of differentially expressed protein between germ-free and
330 conventionally raised mice (Mills et al., 2020). This comparison was made based on the
331 general expectation that genes associated with variation in microbial abundances would
332 be more likely to differ according to the colonization status of the host. Thus, we
333 examined the intersection between genes identified in our study and the proteins
334 identified as highly associated ($|\pi| > 1$) with the colonization state of the colon and the
335 small intestine (Mills et al., 2020). Out of the 373 over- or under-expressed proteins
336 according to colonization status, we find 198 of their coding genes to be among our
337 significant loci, of which 17 are the closest genes to a significant marker (*Iyd*, *Nln*,
338 *Slc26a3*, *Slc3a1*, *Myom2*, *Nebl*, *Tent5a*, *Fxr1*, *Cbr3*, *Chrodc1*, *Nucb2*, *Arhgef10l*, *Sucla2*, *Enpep*,
339 *Prkca*, *Aacs*, and *Cox7c*). This is significantly more than expected by chance (simulated
340 $P = .0156$, 10 000 permutations). Further, analyzing the protein-protein interactions with
341 STRING results in a significant network ($P = 1.73 \times 10^{-14}$, and average node degree 2.4,

342 Suppl. Fig. 11), with *Cyp2c65*, *Cyp2c55*, *Cyp2b10*, *Gpx2*, *Cth*, *Eif3k*, *Eif1*, *Sucla2*, and *Rpl17*
343 identified as hub genes (Suppl. Fig. 12).

344 Subsequently, we merged the information from Mills et al. (2020) and the seven
345 previous QTL mapping studies discussed above to further narrow down the most
346 promising candidate genes, and found 30 genes overlapping with our study. Of these 30
347 genes, six are the closest gene to a significant SNP. These genes are myomesine 2
348 (*Myom2*), solute carrier family 3 member 1 (*Slc3a1*), solute carrier family 26 member 3
349 (*Slc26a3*), nebulin (*Neb1*), carbonyl reductase 3 (*Cbr3*), and acetoacetyl-coA synthetase
350 (*Aacs*).

351 *Candidate genes influencing bacterial abundance*

352 Finally, all previously mentioned candidate genes were combined in one gene set of
353 304 genes and compiled in a highly significant PPI network ($P < 1.0 \times 10^{-16}$, average node
354 degree=4.85, see Methods 4.13). Guided by this network, we filtered out genes situated
355 in the same genomic region and kept the gene with the highest connectivity and
356 supporting information (original network see Suppl. Fig. 13). This gave a resulting gene
357 set of 80 candidate genes (Fig. 6 and Suppl. Table 7). The G protein, GNG12 and the
358 complement component 3 C3, are the proteins with the most edges in the network (30
359 and 25, respectively), followed by MCHR1, CXCL12, and NMUR2 with each 18 edges.
360 Of these 80 highly connected genes, 66 are associated with bacteria that are either co-
361 speciating (co-speciation rate > 0.5 ; Groussin et al., 2017) and/or have high heritability
362 (> 0.5) suggesting a functionally important role for these bacterial taxa (Suppl. Table 7).



363 **Figure 6:** Network of host candidate genes influencing bacterial traits using
364 STRING (<https://string-db.org>). The nodes represent proteins and are colored ac-
365 cording to a selection of enriched GO terms and pathways: G protein coupled re-
366 ceptor (GPCR) signaling (red), regulation of the immune system process (blue), re-
367 sponse to nutrient levels (light green), fatty acid metabolic process (pink), glucose
368 homeostasis (purple), response to antibiotic (orange), regulation of feeding behavior
369 (yellow), positive regulation of insulin secretion (dark green), circadian entrainment
370 (brown), and response to vitamin D (turquoise). The color of the edges represents
371 the interaction type: known interactions from curated databases (turquoise) or ex-
372 perimentally determined (pink); predicted interactions from gene neighborhood
373 (green), gene fusions (red), gene co-occurrence (blue); other interactions from text-
374 mining (light green), co-expression (black), and protein homology (purple).

375 Discussion

376 Understanding the forces that shape variation in host-associated bacterial
377 communities within host species is key to understanding the evolution and maintenance
378 of meta-organisms. Although numerous studies in mice and humans demonstrate that
379 host genetics influences gut microbiota composition (McKnite et al., 2012; Leamy et al.,
380 2014; Goodrich et al., 2014; Org et al., 2015; Davenport et al., 2015; Wang et al., 2016;
381 Bonder et al., 2016; Goodrich et al., 2016; Kemis et al., 2019; Suzuki et al., 2019; Ishida et
382 al., 2020; Hughes et al., 2020; Rühlemann et al., 2021), our study is unique in a number of
383 important ways. First, the unique genetic resource of mice collected from a naturally
384 occurring hybrid zone together with their native microbes yielded extremely high
385 mapping resolution and the possibility to uncover ongoing evolutionary processes in
386 nature. Second, our study is the first to perform genetic mapping of 16S rRNA
387 transcripts in the gut environment, which was previously shown to be superior to DNA-
388 based profiling in a genetic mapping study of the skin microbiota (Belheouane et al.,
389 2017). Third, our study is one of the only to specifically examine the mucosa-associated
390 community. It was previously reasoned that the mucosal environment may better reflect
391 host genetic variation (Spor et al., 2011), and evidence for this hypothesis exists in nature
392 (Linnenbrink et al., 2013). Finally, by cross-referencing our results with previous
393 mapping studies and recently available proteomic data from germ-free versus
394 conventional mice, we curated a more reliable list of candidate genes and pathways.
395 Taken together, these results provide unique and unprecedented insight into the genetic
396 basis for host-microbe interactions.

397 Importantly, by using wild-derived hybrid inbred strains to generate our mapping
398 population, we gained insight into the evolutionary association between hosts and their
399 microbiota at the transition from within species variation to between species divergence.
400 Genetic relatedness in our mapping population significantly correlates with microbiome
401 similarity, supporting a basis for codiversification at the early stages of speciation. A
402 substantial proportion of microbial taxa are heritable, and heritability is correlated with
403 cospeciation rates. This suggests that (i) vertical transmission could enable greater host
404 adaptation to bacteria and/or (ii) the greater number of host genes associated with
405 cospeciating taxa could indicate a greater dependency on the host, such that survival
406 outside a specific host is reduced, making horizontal transmission less likely.

407 By performing 16S rRNA gene profiling at both the DNA and RNA level, we found
408 that 30% (DNA-based) to 45% (RNA-based) of bacterial taxa are heritable, which is
409 consistent with or higher than estimates reported in humans (~10%, Goodrich et al.,
410 2016; ~21%, Turpin et al., 2016) and previous mouse studies (Kovacs et al., 2011; McKnite
411 et al., 2012; Campbell et al., 2012; O'Connor et al., 2014; Carmody et al., 2015; Korach-
412 Rechtman et al., 2019;). The high proportion of heritable taxa, with estimates of up to
413 91%, is likely explained in part by several factors of our study design. First, mice were

414 raised in a controlled common environment, and heritability estimates in other
415 mammals were shown to be contingent on the environment (Grieneisen et al., 2021).
416 Further, bacterial communities were sampled from cecal tissue instead of fecal content
417 (Linnenbrink et al., 2013), and genetic variation was higher than in a typical mapping
418 study due to subspecies differences. For the RNA-based traits, heritability estimates
419 were significantly correlated with previously reported cospeciation rates in mammals
420 (Groussin et al., 2017). This pattern, as well as the higher proportion of heritable taxa in
421 RNA-based traits, suggest that host genetic effects are more strongly reflected by
422 bacterial activity than cell number.

423 Accordingly, we found a total of 179 and 313 unique significant loci for DNA-based
424 and RNA-based bacterial abundance, respectively, passing the conservative study-wide
425 significance threshold. Taxa had a median of five significant loci, suggesting a complex
426 and polygenic genetic architecture affecting bacterial abundances. We identify a higher
427 number of loci in comparison to previous QTL and GWAS studies in mice (Benson et al.,
428 2010; McKnite et al., 2012; Leamy et al., 2014; Wang et al., 2015; Org et al., 2015; Snijders
429 et al., 2016; Kemis et al., 2019), which may be due to a number of factors. The parental
430 strains of our study were never subjected to rederivation and subsequent reconstitution
431 of their microbiota, and natural mouse gut microbiota are more variable than the
432 artificial microbiota of laboratory strains (Kohl and Dearing, 2014; Weldon et al., 2015;
433 Suzuki, 2017; Rosshart et al., 2017;). Furthermore, as noted above, our mapping
434 population harbors both within- and between-subspecies genetic variation. We crossed
435 incipient species sharing a common ancestor ~ 0.5 million years ago, hence we may also
436 capture the effects of mutations that fixed rapidly between subspecies due to strong
437 selection, which are typically not variable within species (Walsh, 1998; Barton and
438 Keightley, 2002).

439 Importantly, our results also help to describe general features of the genetic
440 architecture of bacterial taxon activity. For the majority of loci, the allele associated with
441 lower relative abundance of the bacterial taxon was (partially) dominant. This suggests
442 there is strong purifying selection against a high abundance of any particular taxon,
443 which may help ensure high alpha diversity. The heterozygotes of one-third of
444 significant SNPs displayed transgressive phenotypes. This is consistent with previous
445 studies of hybrids (Turner et al., 2012; Turner and Harr, 2014; Wang et al., 2015;), for
446 example, wild-caught hybrids showed broadly transgressive gut microbiome
447 phenotypes. This pattern can be explained by over- or underdominance, or by epistasis
448 (Rieseberg et al., 1999).

449 Notably, many loci significantly associated with bacterial abundance in this study
450 were implicated in previous studies (Fig. 5). For example, chromosome 2 169-171 Mb is
451 associated with ASV23 (*Eisenbergiella*), *Eisenbergiella* and ASV32 (unclassified
452 Lachnospiraceae) in this study, and overlaps with significant loci from three previous
453 studies (Leamy et al., 2014; Snijders et al., 2016; Kemis et al., 2019). This region contains

454 eight protein-coding genes: *Gm11011*, *Znf217*, *Tshz2*, *Bcas1*, *Cyp24a1*, *Pfdn4*,
455 *4930470P17Rik*, and *Dok5*. Another hotspot is on chromosome 5 101-103 Mb. This locus is
456 significantly associated with four taxa in this study (Prevotellaceae, *Paraprevotella*, ASV7
457 genus *Paraprevotella* and *Acetatifactor*) and overlaps with associations for Clostridiales,
458 Clostridiaceae, Lachnospiraceae, and Deferribacteriaceae (Snijders et al., 2016). Protein-
459 coding genes in this region are: *Nkx6-1*, *Cds1*, *Wdfy3*, *Arhgap24*, and *Mapk10*. As previous
460 studies were based on rederived mouse strains, identifying significant overlap in the
461 identification of host loci suggests that some of the same genes and/or mechanisms
462 influencing major members of gut microbial communities are conserved even in the face
463 of community 'reset' in the context of re-derivation. The identity of the taxa is however
464 not always the same, which suggests that functional redundancy may contribute to
465 these observations, if e.g. several bacterial taxa fulfill the same function within the gut
466 microbiome (Moya and Ferrer, 2016; Tian et al., 2020). Additionally, there is significant
467 overlap of genes within loci identified in the current study and proteins differentially
468 expressed in the intestine of germ-free mice compared to conventionally raised mice
469 (Mills et al., 2020). Finally, by analyzing the functions of the genes closest to significant
470 SNPs, we found that 12 of the 14 significantly enriched KEGG pathways were shown to
471 be related to interactions with bacteria (Fonken et al., 2010; Thaïss et al., 2014; Neumann
472 et al., 2014; Thaïss et al., 2015a; Thaïss et al., 2015b; Castoldi et al., 2015; Erdman and
473 Poutahidis, 2016; Thaïss et al., 2016; Deaver et al., 2018; Wu et al., 2018; Peng et al., 2020;
474 Nagpal et al., 2020; Hollander and Kaunitz, 2020; Suppl. Table 5).

475 To improve the robustness of our results, we combined multiple lines of evidence to
476 prioritize candidates, resulting in a network of 80 genes (Suppl. Table 7). At the center of
477 this network is a set of 22 proteins involved in G-protein coupled receptor signaling (Fig.
478 6, red nodes). MCHR1, NMUR2, and TACR3 (Fig. 6, yellow) are known to regulate
479 feeding behavior (Saito et al., 1999; Cardoso et al., 2012; Smith et al., 2019), and CHRM3
480 to control digestion (Gautam et al., 2006; Tanahashi et al., 2009). Gut microbes can
481 produce GPCR agonists to elicit host cellular responses (Cohen et al., 2017; Colosimo et
482 al., 2019; Chen et al., 2019; Pandey et al., 2019). Thus, GPCRs may be key modulators of
483 communication between the gut microbiota and host. Another interesting group of
484 genes are those responding to nutrient levels (*Bmp7*, *Cd40*, *Aacs*, *Gclc*, *Nmur2*, *Cyp24a1*,
485 *Adcyap1*, *Serpinc1*, and *Wnt11*) (Sethi and Vidal-Puig, 2008; Peier et al., 2009; Townsend et
486 al., 2012; Yi and Bishop, 2015; Shi and Tu, 2015; Toderici et al., 2016; Yasuda et al., 2021;
487 Gastelum et al., 2021;), as gut microbiota affect host nutrient uptake (Chung et al., 2018).
488 In addition, CYP24A1, BMP7 and CD40 respond to vitamin D. Previous studies
489 identified vitamin D/the vitamin D receptor to play a role in modulating the gut
490 microbiota (Wang et al., 2016; Malaguarnera, 2020; Yang et al., 2020b; Singh et al., 2020),
491 and CD40 is known to induce a vitamin D dependent antimicrobial response through
492 IFN- γ activation (Klug-Micu et al., 2013).

493 Another important category of candidate genes are those involved in immunity.
494 Our most significant SNP was situated downstream of the *Tlr4* gene and was associated
495 with the genus *Dorea* and several *Dorea* species. *Dorea* is a known short chain fatty acid
496 producer (Taras et al., 2002; Reichardt et al., 2018) and interacts with tight junction
497 proteins *Claudin-2* and *Occludin* (Alhasson et al., 2017). *Tlr4* is a member of the Toll-like
498 receptor family, and has been linked with obesity, inflammation, and changes in the gut
499 microbiota (Velloso et al., 2015). These combined results reflect an important role for
500 *Dorea* in fatty acid harvesting and intestinal barrier integrity, both of which could act
501 systemically to activate TLR4 and to promote metabolic inflammation (Cani et al., 2008;
502 Delzenne et al., 2011; Nicholson et al., 2012). Moreover, the SNP with the second lowest
503 *P* value was associated with the same taxa and situated 181 kb upstream of *Irak4*. IRAK4
504 is rapidly recruited after TLR4 activation to enable downstream activation of the NFκB
505 immune pathway. *Irak4* has previously been associated with a change in bacterial
506 abundance using inbred mice (McKnite et al., 2012; Org et al., 2015).

507 Finally, we identified notable links between candidate genes and five human
508 diseases (mental disorders, blood pressure finding, systemic arterial pressure, substance-
509 related disorders, and atrial septal deficits; Suppl. Fig. 10). The connection to mental
510 disorders is intriguing as involvement of the gut microbiota is suspected (Kelly et al.,
511 2015; Foster et al., 2017; Cox and Weiner, 2018; Chen et al., 2019; Sarkar et al., 2020;
512 Parker et al., 2020; Flux and Lowry, 2020). Taken together with our finding of an
513 enriched set of GPCRs, this highlights the importance of host-microbial interplay along
514 the gut-brain axis. Moreover, we also identify a significant over-representation of IBD
515 genes (Khan et al., 2021) among the 925 genes nearest to significant SNPs (Suppl. Table
516 6). Interestingly, SNPs in five out of 14 genes are associated with ASVs belonging to the
517 genus *Oscillibacter*, a highly cospeciating taxon known to decrease during the active state
518 of IBD (Metwaly et al., 2020).

519 In summary, our study provides a number of novel insights into the importance of
520 host genetic variation in shaping the gut microbiome, in particular for cospeciating
521 bacterial taxa. These findings provide an exciting foundation for future studies of the
522 precise mechanisms underlying host-gut microbiota interactions in the mammalian gut
523 and should encourage future genetic mapping studies that extend analyses to the
524 functional metagenomic sequence level.

525 **Materials and Methods**

526 *Intercross design*

527 We generated a mapping population using partially inbred strains derived from
528 mice captured in the *M. m. musculus* - *M. m. domesticus* hybrid zone around Freising,
529 Germany in 2008 (Turner et al., 2012). Originally, four breeding stocks were derived
530 from 8-9 ancestors captured from one (FS, HA, TU) or two sampling sites (HO), and
531 maintained with four breeding pairs per generation using the HAN-rotation out-
532 breeding scheme (Rapp, 1972). Eight inbred lines (two per breeding stock) were
533 generated by brother/sister mating of the 8th generation lab-bred mice. We set up the
534 cross when lines were at the 5th-9th generation of brother-sister meeting, with
535 inbreeding coefficients of > 82%.

536 We first set up eight G1 crosses, each with one predominantly *domesticus* line (FS,
537 HO - hybrid index <50%; see below) and one predominantly *musculus* line (HA, TU -
538 hybrid index >50%); each line was represented as a dam in one cross and sire in another
539 (Suppl. Fig. 14). One line, FS5, had a higher hybrid index than expected, suggesting
540 there was a misidentification during breeding (see genotyping below). Next, we set up
541 G2 crosses in eight combinations (subcrosses), such that each G2 individual has one
542 grandparent from each of the initial four breeding stocks. We included 40 males from
543 each subcross in the mapping population.

544 This study was performed according to approved animal protocols and in-
545 stitutional guidelines of the Max Planck Institute. Mice were maintained and handled in
546 accordance with FELASA guidelines and German animal welfare law (Tierschutzgesetz
547 § 11, permit from Veterinäramt Kreis Plön: 1401-144/PLÖ-004697).

548 *Sample collection*

549 Mice were sacrificed at 91 ± 5 days by CO₂ asphyxiation. We recorded body weight,
550 body length and tail length, and collected ear tissue for genotyping. The caecum was
551 removed and gently separated from its contents through bisection and immersion in
552 RNAlater (Thermo Fisher Scientific, Schwerte, Germany). After overnight storage in
553 RNAlater at 4° C, the RNAlater was removed and tissue stored at -20° C.

554 *DNA extraction and sequencing*

555 We simultaneously extracted DNA and RNA from caecum tissue samples using
556 Qiagen (Hilden, Germany) Allprep DNA/RNA 96-well kits. We followed the
557 manufacturer's protocol, with the addition of an initial bead beating step using Lysing
558 matrix E tubes (MP Biomedical, Eschwege) to increase cell lysis. We used caecum tissue
559 because host genetics has a greater influence on the microbiota at this mucosal site than
560 on the lumen contents (Linnenbrink et al., 2013). We performed reverse transcription of
561 RNA with High-Capacity cDNA Transcription Kits from Applied Biosystems
562 (Darmstadt, Germany). We amplified the V1-V2 hypervariable region of the 16S rRNA

563 gene using barcoded primers (27F-338R) with fused MiSeq adapters and heterogeneity
564 spacers following (Rausch et al., 2016) and sequenced amplicons with 250 bp paired-
565 reads on the Illumina MiSeq platform.

566 *16S rRNA gene analysis*

567 We assigned sequences to samples by exact matches of MID (multiplex identifier, 10
568 nt) sequences processed 16S rRNA sequences using the DADA2 pipeline, implemented
569 in the DADA2 R package, version 1.16.0 (Callahan et al., 2016; Callahan, 2016). Briefly,
570 raw sequences were trimmed and quality filtered with the maximum two 'expected
571 errors' allowed in a read, paired sequences were merged and chimeras removed. For all
572 downstream analyses, we rarefied samples to 10,000 reads each. Due to the quality
573 filtering, we have phenotyping data for 286 individuals on DNA level, and 320 G2
574 individuals on RNA level. We classified taxonomy using the Ribosomal Database Project
575 (RDP) training set 16 (Cole et al., 2014). Classifications with low confidence at the genus
576 level (<0.8) were grouped in the arbitrary taxon 'unclassified_group'.

577 We used the phyloseq R package (version 1.32.0) to estimate alpha diversity using
578 the Shannon index and Chao1 index, and beta diversity using Bray-Curtis distance
579 (McMurdie and Holmes, 2013). We defined core microbiomes at the DNA- and RNA-
580 level, including taxa present in > 25% of the samples and with median abundance of
581 non-zero values > 0.2% for amplicon sequence variant (ASV) and genus; and >0.5% for
582 family, order, class and phylum.

583 *Genotyping*

584 We extracted genomic DNA from ear samples using Qiagen Blood and Tissue 96
585 well kits (Hilden, Germany), according to the manufacturer's protocol. We sent DNA
586 samples from 26 G0 mice and 320 G2 mice to GeneSeek (Neogen, Lincoln, NE) for
587 genotyping using the Giga Mouse Universal Genotyping Array (GigaMUGA; Morgan et
588 al., 2015), an Illumina Infinium II array containing 141,090 single nucleotide
589 polymorphism (SNP) probes. We quality-filtered genotype data using plink 1.9 (Chang
590 et al., 2015); we removed individuals with call rates <90% and SNPs that were: not bi-
591 allelic, missing in >10% individuals, with minor allele frequency <5%, or Hardy-
592 Weinberg equilibrium exact test P values <1e-10. A total of 64,103 SNPs and all but one
593 G2 individual were retained. Prior to mapping, we LD-filtered SNPs with $r^2 > 0.9$ using a
594 window of 5 SNPs and a step size of 1 SNP. We retain 32,625 SNPs for mapping.

595 *Hybrid index calculation*

596 For each G0 and G2 mouse, we estimated a hybrid index – defined as the
597 percentage of *M. m. musculus* ancestry. We identified ancestry-informative SNP markers
598 by comparing GigaMUGA data from ten individuals each from two wild-derived
599 outbred stocks of *M. m. musculus* (Kazakhstan and Czech Republic) and two of *M. m.*
600 *domesticus* (Germany and France) maintained at the Max Planck Institute for

601 Evolutionary Biology (L.M. Turner and B. Payseur, unpublished data). We classified
602 SNPs as ancestry informative if they had a minimum of 10 calls per subspecies, the
603 major allele differed between *musculus* and *domesticus*, and the allele frequency
604 difference between subspecies was > 0.3 . A total of 48,361 quality-filtered SNPs from the
605 G0/G2 genotype data were informative, including 8,775 SNPs with fixed differences
606 between subspecies samples.

607 *Correlation between host relatedness and microbiome structure*

608 To investigate if host relatedness is correlated with individual variation in
609 microbiome composition, we computed a centered relatedness matrix using the 32,625
610 filtered SNPs with GEMMA (v 0.98.1; Zhou and Stephens, 2012) and microbial
611 composition-based kinship matrix among individuals based on relative bacterial
612 abundances (Chen et al., 2018). The kinship matrix was calculated with the formula:

$$Kinship = 1/p \sum_{i=1}^p (x_i - 1_n \bar{x}_i)(x_i - 1_n \bar{x}_i)^T$$

613 where X denotes the $n \times p$ matrix of genotypes or relative abundances, x_i as its i th
614 column representing the genotypes of i th SNP or the relative abundance of the i th ASV,
615 \bar{x}_i as the sample mean and 1_n as a $n \times 1$ vector of 1's. We used a Mantel test with the
616 Spearman's correlation to test for correlation between the host SNP-based kinship and
617 microbial composition-based kinship using 10,000 permutations.

618 *SNP-based heritability of microbial abundances*

619 We calculated SNP-based heritabilities for bacterial abundances using a linear
620 mixed model implemented in the lme4qtl R package (version 0.2.2; Ziyatdinov et al.,
621 2018). The SNP-based heritability is expressed as:

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_m^2 + \sigma_e^2}$$

622 where σ_g^2 is the genetic variance estimated by K_{SNP} , σ_m^2 variance of the mating pair
623 component, and σ_e^2 the variance due to residual environmental factors. We determined
624 significance of the heritability estimates using exact likelihood ratio tests, following
625 Supplementary Note 3 in Ziyatdinov et al., 2018, using the exactLRT() function of the R
626 package RLRsim (version 3.1-6; Fabian et al., 2008).

627 *Genome-wide association mapping*

628 Prior to mapping, we inverse logistic transformed bacterial abundances using the
629 inv.logit function from the R package gtools (version 3.9.2; Gregory R. Warnes, 2020).

630 We performed association mapping in the R package lme4qtl (version 0.2.2;
631 Ziyatdinov et al., 2018) with the following linear mixed model:

$$y_i = \mu + a_i X_{ij}^a + d_i X_{ij}^d + Wu + e$$

632

633 where y_j is the phenotypic value of the j th individual; μ is the mean, X_{nij} the additive
634 and X_{dij} the dominance genotypic index values coded as for individual j at locus i . a and
635 d indicate fixed additive and dominance effects, W indicates random effects mating pair
636 and kinship matrix, plus residual error e .

637 We estimated additive and dominance effects separately because we expected to
638 observe underdominance and overdominance in our hybrid mapping population, as
639 well as additive effects, and aimed to estimate their relative importance. To model the
640 additive effect (i.e. 1/2 distance between homozygous means), genotypes at each locus,
641 i , were assigned additive index values ($X^a \in 1, 0, -1$) for AA, AB, BB, respectively, with A
642 indicating the major allele and B the minor allele. To model dominance effects (i.e.
643 heterozygote mean - midpoint of homozygote means), genotypes were assigned
644 dominance index values ($X^d \in 0, 1$) for homozygotes and heterozygotes, respectively.

645 We included mating pair as a random effect to account for maternal effects and cage
646 effects, as male litter mates are kept together in a cage after weaning. We included
647 kinship coefficient as a random effect in the model to account for population and family
648 structure. To avoid proximal contamination, we used a leave-one-chromosome-out
649 approach, that is, when testing each single-SNP association we used a relatedness matrix
650 omitting markers from the same chromosome (Parker et al., 2014). Hence, for testing
651 SNPs on each chromosome, we calculated a centered relatedness matrix using SNPs
652 from all other chromosomes with GEMMA (v0.97; Zhou and Stephens, 2012). We
653 calculated P values for single-SNP associations by comparing the full model to a null
654 model excluding fixed effects. Code for performing the mapping is available at [https://](https://github.com/sdoms/mapping_scripts)
655 github.com/sdoms/mapping_scripts.

656 We evaluated significance of SNP-trait associations using two thresholds; first, we
657 used a genome-wide threshold for each trait, where we corrected for multiple testing
658 across markers using the Bonferroni method (Abdi, 2007). Second, as bacteria interact
659 with each other within the gut as members of a community, bacterial abundances are
660 non-independent, so we calculated a study-wide threshold dividing the genome-wide
661 threshold by the number of effective taxa included. We used matSpDlite (Nyholt, 2019;
662 Li and Ji, 2005; Qin et al., 2020) to estimate the number of effective bacterial taxa based
663 on eigenvalue variance.

664 To estimate the genomic interval represented by each significant LD-filtered SNP,
665 we report significant regions defined by the most distant flanking SNPs in the full pre-
666 LD-filtered genotype dataset showing $r^2 > 0.9$ with each significant SNP. We combined
667 significant regions less than 10 Mb apart into a single region. Genes situated in

668 significant regions were retrieved using biomaRt (Steffen Durinck, 2009), and the mm10
669 mouse genome.

670 *Dominance analyses*

671 We classified dominance for SNPs with significant associations on the basis of the
672 d/a ratio (Falconer, 1996) where d is the dominance effect, a the additive effect. As the
673 expected value under purely additive effects is 0. As our mapping population is a multi-
674 parental-line cross, and not all SNPs were ancestry-informative with respect to *musculus/*
675 *domesticus*, the sign of a effects is defined by the major allele within our mapping
676 population, which lacks clear biological interpretation. To provide more meaningful
677 values, we report $d/|a|$, such that a value of 1 = complete dominance of the allele
678 associated with higher bacterial abundance, and a value of -1 = complete dominance of
679 the allele associated with lower bacterial abundance. Values above 1 or below -1 indicate
680 over/underdominance. We classified effects of significant regions the following
681 arbitrary $d/|a|$ ranges to classify dominance of significant regions (Burke et al., 2002;
682 Miller et al., 2014): underdominant <-1.25 , high abundance allele recessive between -1.25
683 and -0.75, partially recessive between -0.75 and -0.25, additive between -0.25 and 0.25,
684 partially dominant between 0.25 and 0.75, dominant 0.75 and 1.25, and
685 overdominant >1.25 .

686 *Gene ontology and network analysis*

687 The nearest genes up- and downstream of the significant SNPs were identified
688 using the locateVariants() function from the VariantAnnotation R package (version
689 1.34.0; Valerie et al., 2014) using the default parameters. A maximum of two genes per
690 locus were included (one upstream, and one downstream of a given SNP).

691 To investigate functions and interactions of candidate genes, we calculated a
692 protein-protein interaction (PPI) network with STRING version 11 (Szklarczyk et al.,
693 2019), on the basis of a list of the closest genes to all SNPs with significant trait
694 associations. We included network edges with an interaction score >0.9 , based on
695 evidence from fusion, neighborhood, co-occurrence, experimental, text-mining,
696 database, and co-expression. We exported this network to Cytoscape v 3.8.2 (Shannon et
697 al., 2003) for identification of highly interconnected regions using the MCODE
698 Cytoscape plugin (Bader and Hogue, 2003), and functional annotation of clusters using
699 the stringApp Cytoscape plugin (Doncheva et al., 2019).

700 We identified overrepresented KEGG pathways and human diseases using the
701 clusterProfiler R package (version 3.16.1; Yu et al., 2012). P values were corrected for
702 multiple testing using the Benjamini-Hochberg method. Pathways and diseases with an
703 adjusted P value $< .05$ were considered over-represented.

704 *Calculating overlap with other studies and over-representation of IBD genes*

705 To test for significant overlap with loci identified in previous mapping studies and
706 for over-representation of IBD genes, we used the tool *poverlap* (Brent Pedersen, 2013) to
707 compare observed overlap to random expectations based on 10,000 permutations of
708 significant regions. We identified genes within overlapping regions using the
709 `locateVariants()` function from the VariantAnnotation R package (version 1.34.0; Valerie
710 et al., 2014).

711 *Combination of results*

712 Hub genes SNP network and their first neighbors, the hub genes from the
713 ‘differentially expressed in GF mice’-network and their respective first neighbors, genes
714 found in both Mills et al. (2020) and other mouse QTL studies, closest genes to a SNP
715 found in Mills et al. (2020), genes situated in the 20 smallest intervals, six genes in the
716 two intervals with the lowest *P* values, twenty genes in intervals found in most different
717 taxa, genes situated in the region with most overlap within our study, and finally the
718 genes situated in the intervals that most frequently overlapped with other studies were
719 combined into one gene set and analyzed with STRING. Genes situated in the same
720 genomic locus were curated according to the number of edges in the STRING network.

721 **Data and code availability:** DNA- and RNA-based 16S rRNA gene sequences are
722 available under project accession number PRJNA759194. Code is available at [https://](https://github.com/sdoms/mapping_scripts)
723 [/github.com/sdoms/mapping_scripts](https://github.com/sdoms/mapping_scripts).

724 **Supplementary Materials:** Suppl. Fig 1-14, Suppl. Table 1: Heritability estimates,
725 Suppl. Table 2: Genome-wide significant associations, Suppl. Table 3: Study-wide
726 significant associations, Suppl. Table 4: Intervals smaller than 1Mb, Suppl. Table 5:
727 Over-represented KEGG pathways, Suppl. Table 6: IBD genes, Suppl. Table 7:
728 Candidate genes.

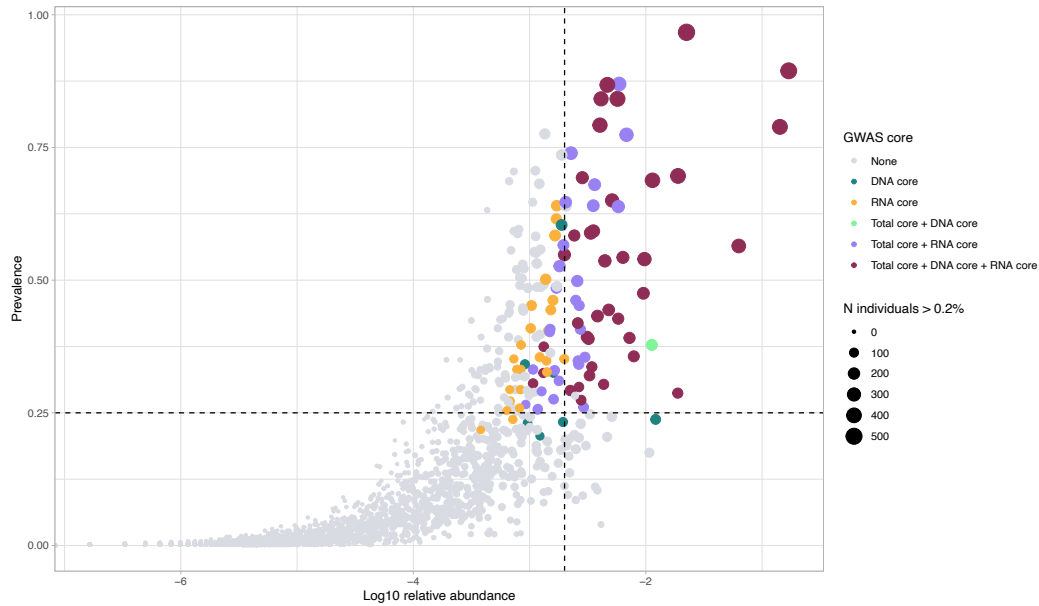
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737 **Author contributions:** Conceptualization: L.M.T., S.I., A.F., and J.F.B; Methodology:
738 L.M.T, J.F.B., S.D., S.I., A.F., and A.K.; Software: S.D., M.R., and L.M.T; Validation: S.D.,
739 M.R., A.K., and L.M.T.; Formal Analysis: S.D., M.R., A.K., and L.M.T; Investigation: S.D.
740 and L.M.T.; Resources: S.D., H.F., and C.C; Writing - Original Draft: S.D.; Writing -

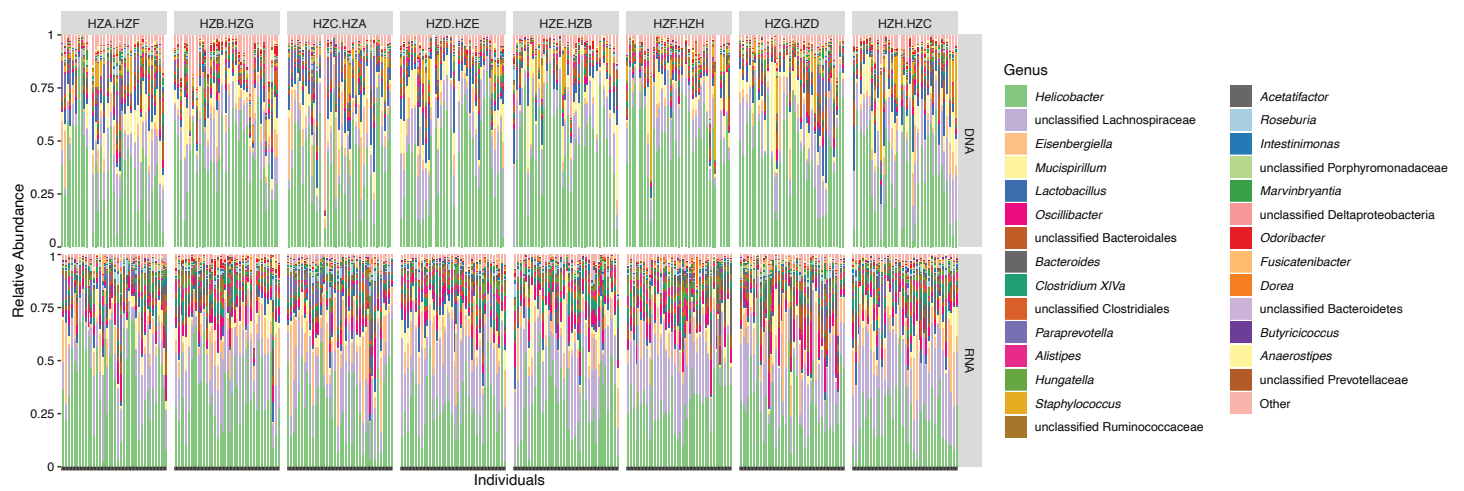
741 Review & Editing: S.D., L.M.T, and J.F.B; Visualization: S.D. and L.M.T; Supervision:
742 L.M.T, A.F, and J.F.B.; Project Administration: J.F.B.; Funding Acquisition: A.F, L.M.T,
743 and J.F.B.

744 **Conflicts of Interest: The authors declare no conflict of interest.**

745 Supplementary figures



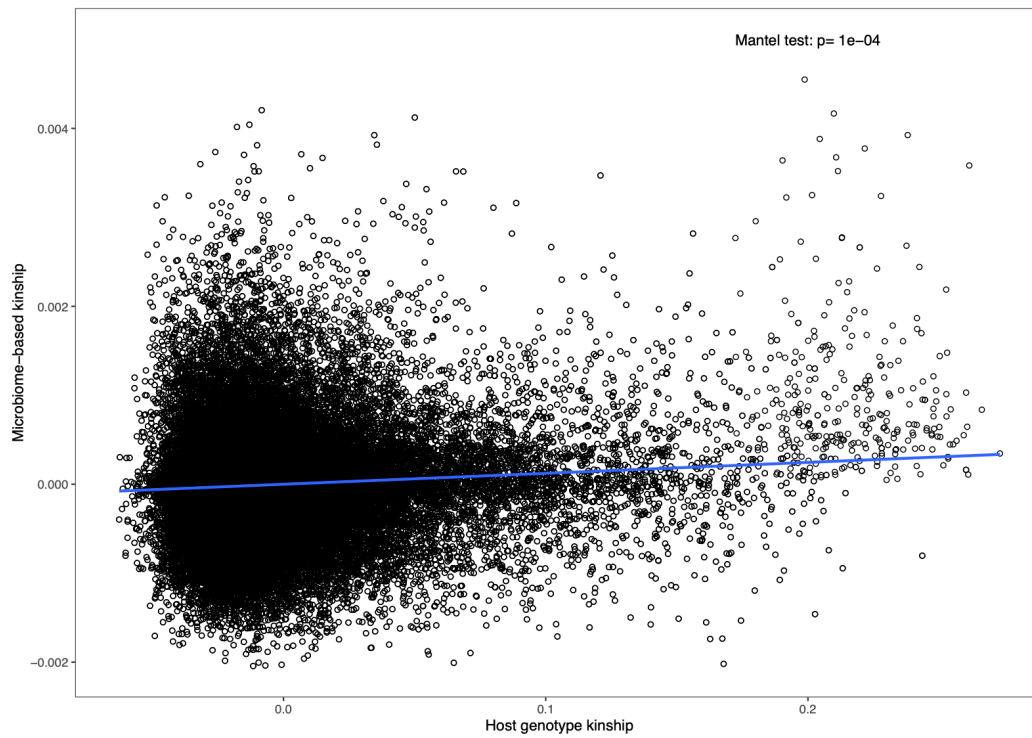
746 **Supplementary figure 1:** Selection of taxa for mGWAS analysis. A scatter plot showing the
747 association of average relative abundance of taxa with their prevalence in the G2 mapping
748 population. Taxa retained for analysis are colored according to the originating core. The size of
749 each dot represents the number of individuals that have a median abundance higher than 0.2% of
750 the taxon. The dashed lines represent the thresholds of the core (vertical: median abundance > 0.2%
751 and horizontal prevalence of 25 %).



752 **Supplementary figure 2: Relative abundances of core genera in G2 mapping population.**

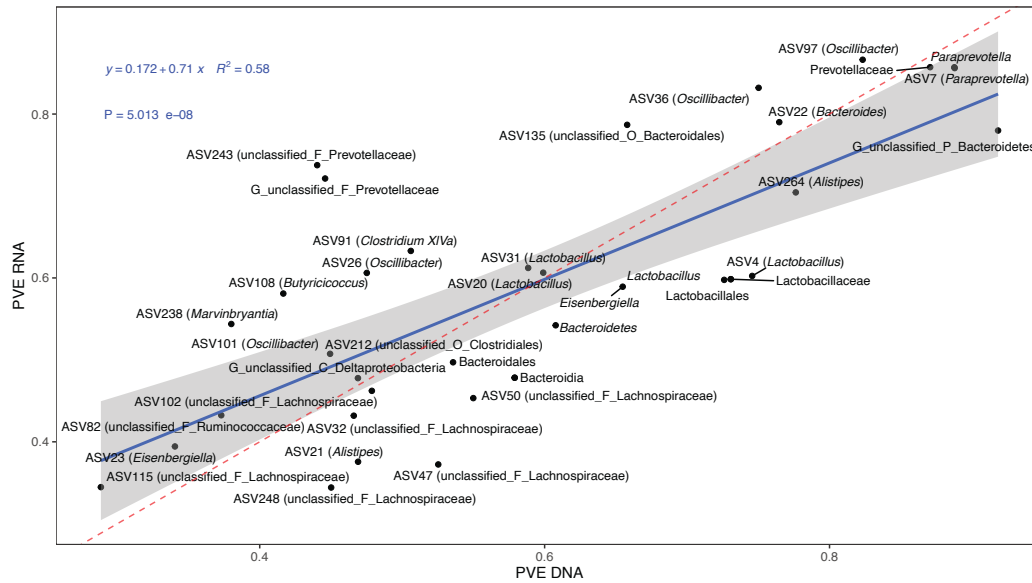
753 Each vertical line represents one individual. Subcross (see supplementary figure 14) is indicated at

754 the top.



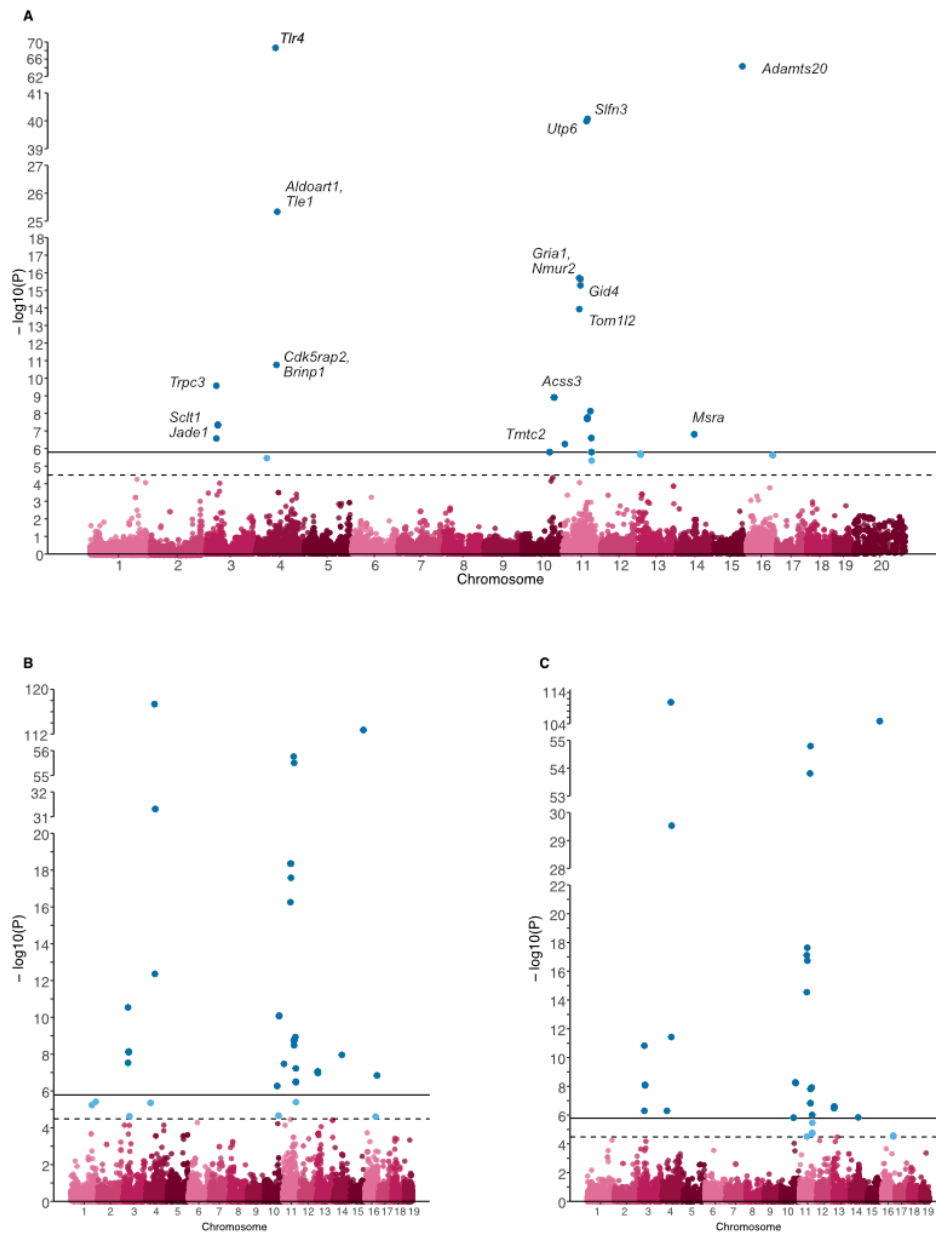
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Supplementary figure 3: Host genetic relatedness calculated from SNP data (x-axis) correlated with microbial composition-based relatedness (y-axis) calculated from ASV abundances. The blue line represents a linear regression fit to the data.



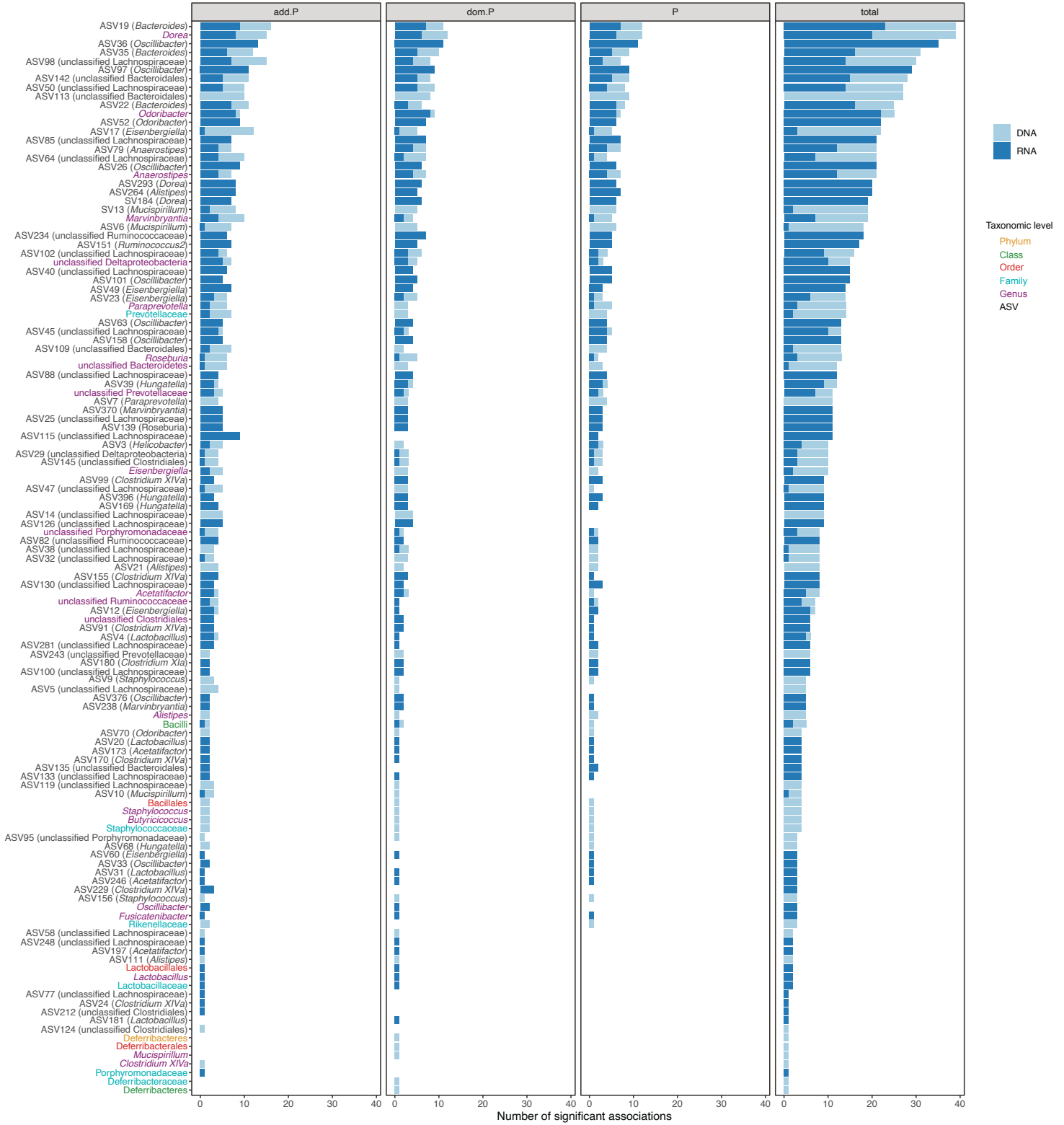
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Supplementary figure 4: Correlation of SNP-based heritability estimates based on DNA (x-axis) or RNA (y-axis). The blue line represents a linear regression fit to the data. Red dashed line represents the identity line with a slope of 1.



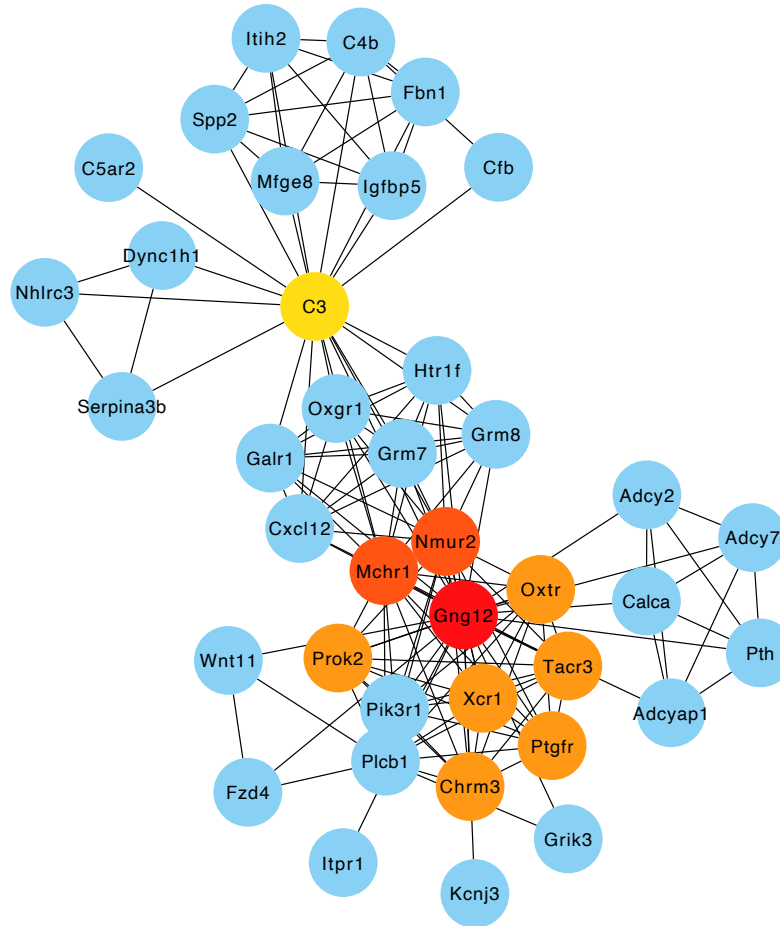
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Supplementary figure 5: Manhattan plots for ASV184 (*Dorea*) of the complete model (A), the additive effect (B) or the dominance effect (C). SNPs passing the study-wide significance threshold (solid line) are shown in dark blue, while genome-wide significant SNPs (dashed line) are shown in light blue. In panel A, the closest gene to the SNP is shown for a subset of significant SNPs.



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Supplementary figure 6: Number of significantly associated loci per bacterial taxon. Loci with significant additive effects (add.P), dominance effects (dom.P) or effects in full model (P) are indicated.



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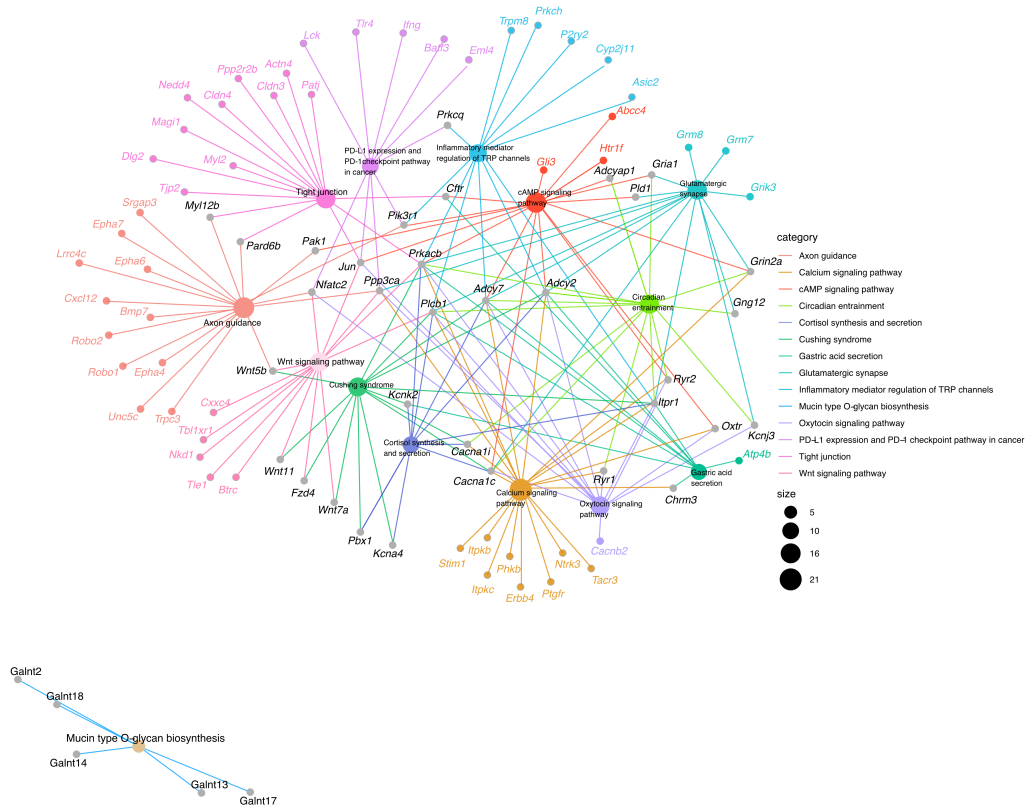
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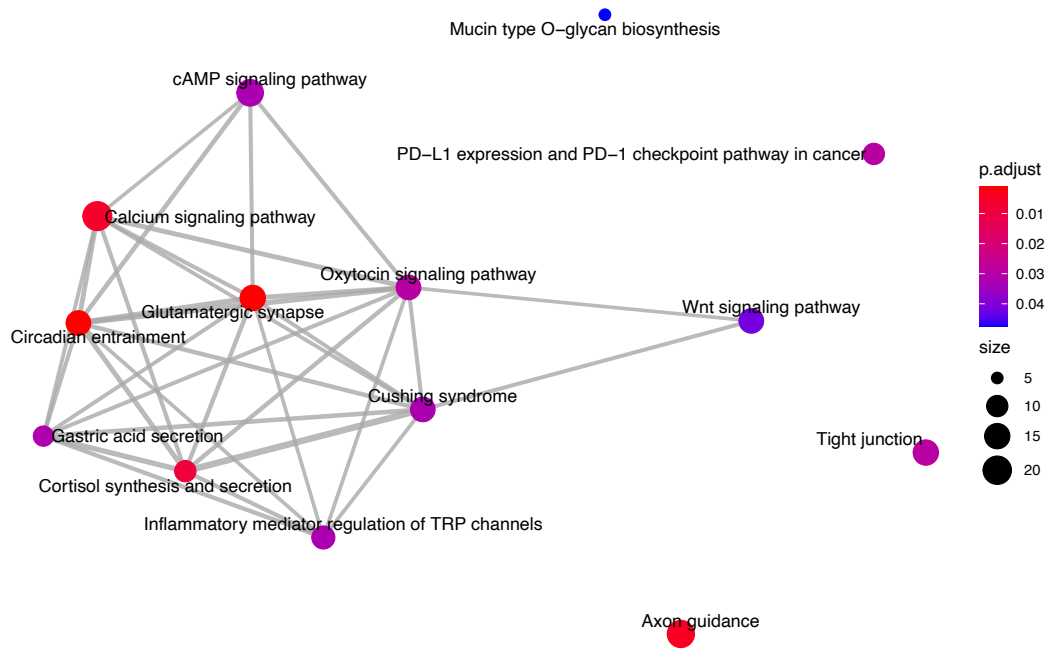
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Supplementary figure 7: Top ten hub genes of the protein-protein interaction (PPI) network with the closest genes to the host SNPs significantly associated with bacterial abundances. The nodes are colored according to hub gene rank from 1 (red) to 10 (yellow). Blue nodes are the first neighbors.

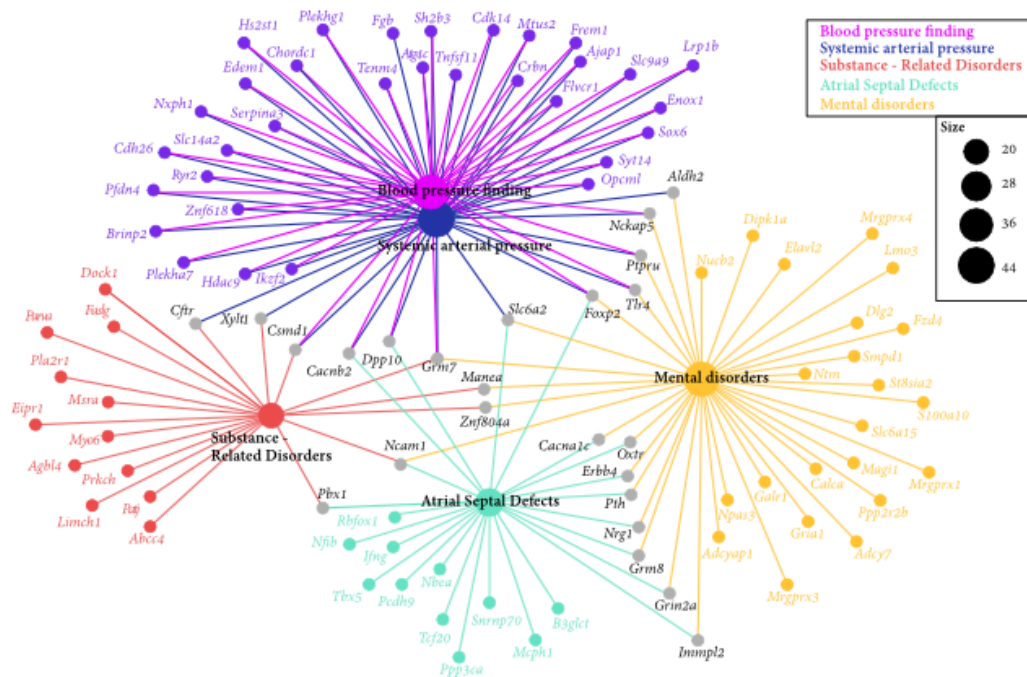


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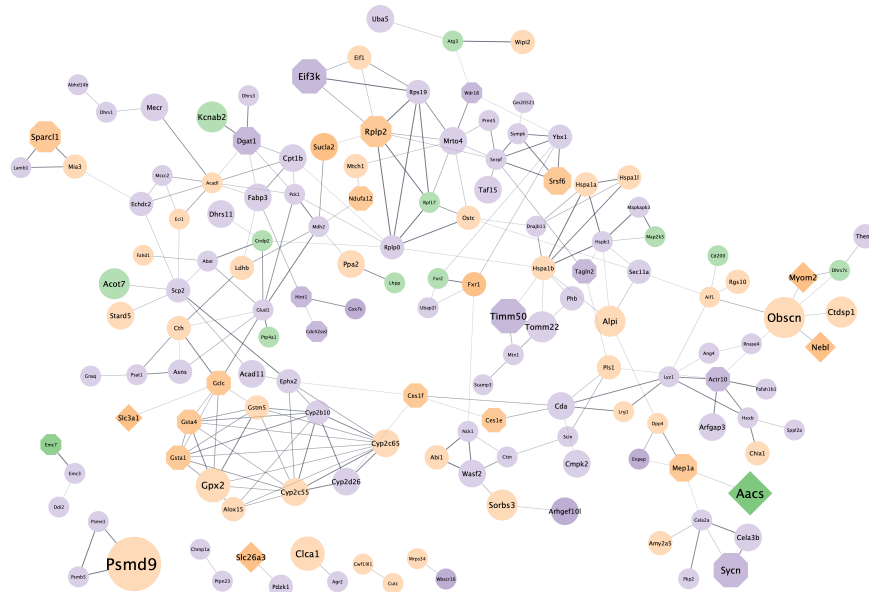
Supplementary figure 8: Genes belonging to over-represented KEGG pathways within the host genes closest to significant SNPs from association analysis.



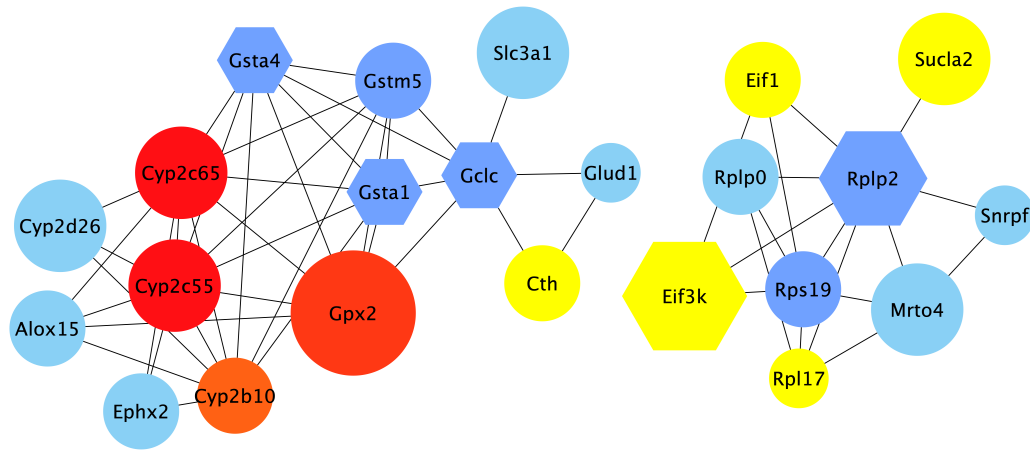
776 **Supplementary figure 9:** Enriched KEGG pathways among closest genes to significant SNPs from
777 association analysis. Node color indicates FDR-adjusted P value of enrichment and node size indi-
778 cates number of candidate genes in pathway.



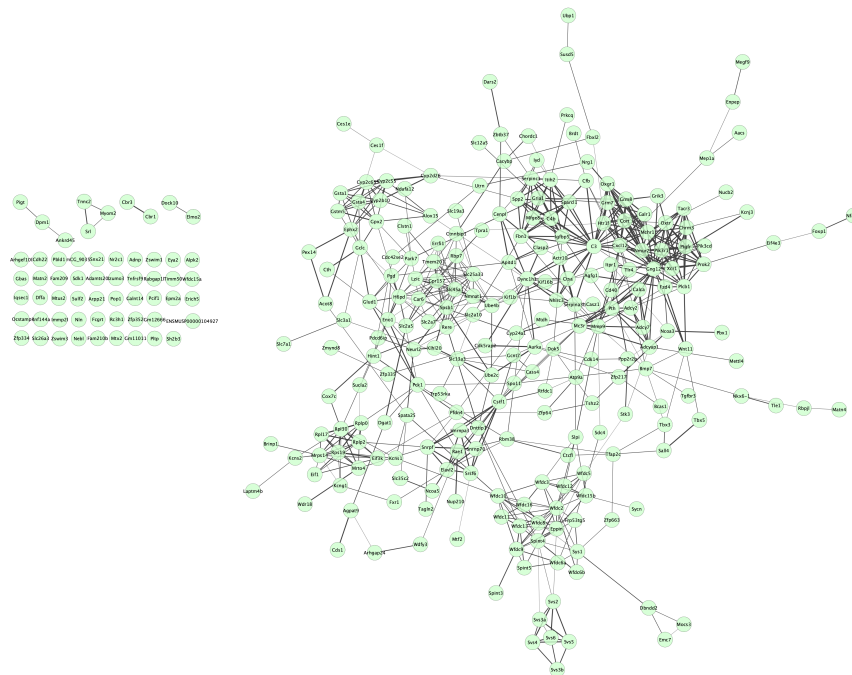
779 **Supplementary figure 10:** Enriched human diseases among genes closest to significant SNPs from
780 association analysis.



781 **Supplementary figure 11:** STRING (Szklarczyk et al., 2019) protein-protein interaction network of
782 proteins that are differentially expressed in the intestine (small intestine and colon) of germ-free
783 (GF) mice compared to conventionally raised mice, found in the present study. The color of the
784 network nodes indicates whether the QTL hit was found using the DNA abundances (green),
785 RNA abundances (purple) or was found in both (orange). The shape represents if the gene of the
786 protein was the closest gene to the significant SNP (rectangle), if the gene was also found in QTLs
787 of other studies (octagon), a combination of both (diamond), or only differentially expressed in GF
788 mice vs. conventionally raised mice. The node size expresses the number of taxa where the gene
789 was found in a QTL. The edges represent protein-protein interactions, where the line thickness in-
790 dicates the strength of the data support from text mining, experiments, databases, co-expression,
791 gene-fusion, and co-occurrence.

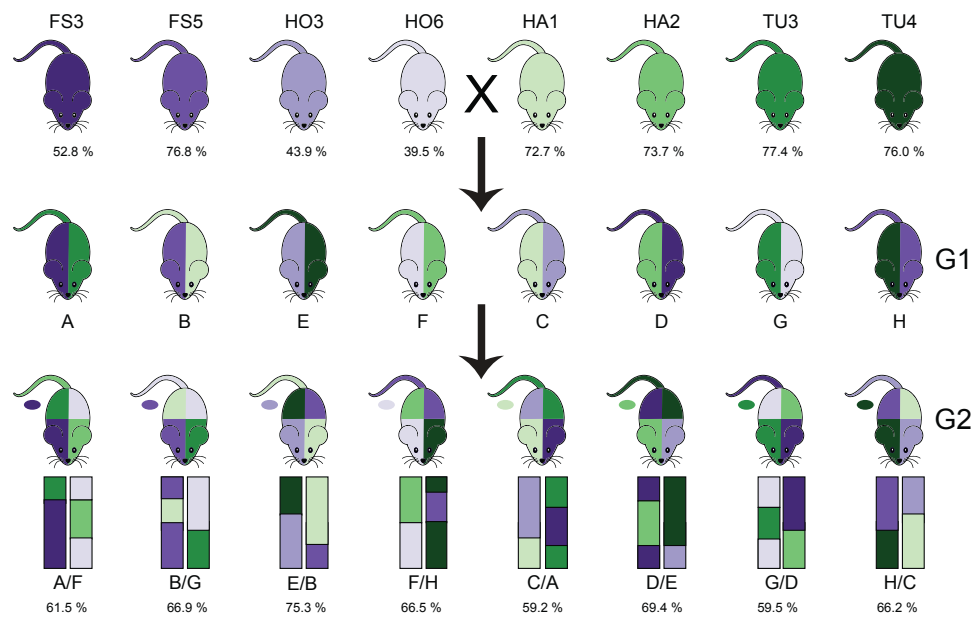


792 **Supplementary figure 12:** Visualization of the top hub genes calculated with the MCC algo-
793 rithm and their first neighbors from the protein-protein interaction (PPI) network of genes
794 found in intervals in present study that are also differentially expressed in germ-free versus
795 conventionally raised mice. Edges represent the protein-protein associations. The red nodes
796 represent genes with a high degree (= hub genes), and the yellow nodes with a low degree,
797 while the blue nodes represent their first neighbors. All nodes shown are differentially ex-
798 pressed in GF mice. Hexagon shaped nodes are genes/proteins also found associated with
799 gut microbiome abundances in other mouse QTL studies, and round nodes are 'only' differ-
800 entially expressed in GF mice. The size of the node is an indication of the amount of taxa as-
801 sociated with the gene.



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Supplementary figure 13: Original protein protein interaction (PPI) network of 304 candidate genes closest to SNPs significantly associated with bacterial abundances. Generated in STRING (Szklarczyk et al., 2019) and Cytoscape (Shannon et al., 2003).



805 **Supplementary figure 14:** Overview of the intercross design. G0 mice are from eight partially
 806 inbred lines derived from mice wild-caught in four hybrid zone sites. Hybrid index - the per-
 807 centage of *musculus* alleles - is reported as the mean for the G0 mice from each line (top), or
 808 mean of 40 G2s from each subclass (bottom). We performed eight G1 crosses with one line
 809 with hybrid index ~50% (purple shades) and one line with hybrid index >50% (green
 810 shades); color on the left side of mouse diagram indicates dam line and right side indicates
 811 sire line. Next, G1 mice were crossed in eight combinations such that each G2 mouse had one
 812 grandparent from each of the four breeding stocks, indicated by colors of mouse diagram,
 813 and representative chromosomes below. Tail color indicates Y chromosome strain, and oval
 814 indicates mitochondrial strain.

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