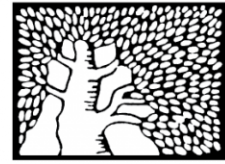


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Structural variation in the gut microbiome associates with host health

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1 **Sub-genomic variation in the gut microbiome associates with host metabolic health**

2

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25

26

27 **Abstract**

28 Differences in the presence of even a few genes between otherwise identical bacterial strains
29 may result in critical phenotypic differences, yet exploring variation at this sub-genomic level
30 across gut microbiomes is challenging, possibly owing to difficulties in correct metagenomic
31 read assignment. Here, we devised algorithms that improve the assignment accuracy of
32 metagenomic reads to reference sequences and systematically identify variability in microbial
33 sub-genomic regions. We find Sub-Genomic Variation (SGV) to be prevalent in the microbiome
34 across multiple phyla, and that our method produces SGVs that replicate across distinct human
35 cohorts from different continents. SGVs are associated with bacterial fitness and their member
36 genes are enriched for CRISPR-associated and antibiotic producing functions and depleted
37 from housekeeping genes, suggestive of a role in microbial adaptation. We find 124 novel
38 associations between SGVs and host disease risk factors, of which 40 replicate in an
39 independent cohort, highlighting the universality of these associations. Finally, by exploring
40 genes clustered in the same SGV, we uncover several possible mechanistic links between the
41 microbiome and its host, as in the case of a 31kbp region in *Anaerostipes hadrus* encoding a
42 composite inositol catabolism-butyrate biosynthesis pathway, whose presence is associated
43 with significantly lower host body weight and metabolic disease risk. Overall, our results uncover
44 a nascent layer of variability in the microbiome that is associated with microbial adaptation and
45 host health.

46

47

48 **Introduction**

49

50 Genes that are deleted or duplicated within different members of a species (also termed copy
51 number variation; CNV), are a phenomenon common across all kingdoms^{1,2}. In humans, CNVs
52 allowed adaptation to starch consumption by an increase in the copy number of the alpha-
53 amylase gene³, and they are also linked to multiple conditions such as autism spectrum
54 disorders⁴, psychiatric disorders⁵, obesity⁶, and autoimmune disease^{7,8}. In bacteria, even a
55 small number of genes can underlie phenotypes such as virulence^{9,10}, antibiotic resistance¹¹,
56 host metabolic disease¹² and even host longevity¹³, making genetic variation highly important to
57 both the microbe and its host.

58 Microbes in the human intestines share copious genetic material¹⁴, resulting in a high
59 prevalence of CNVs across the gut microbiome¹⁵. This variability could be critical to human
60 pathophysiology, as gut microbes were found to be involved in multiple host processes, such as
61 fiber metabolism¹⁶, bile acid metabolism¹⁷, vitamin biosynthesis¹⁸ and immune conditioning¹⁹,
62 and are associated with multiple host disorders ranging from obesity and diabetes^{20,21}, through
63 inflammatory bowel diseases^{22,23}, to macular degeneration²⁴ and autism²⁵. The mechanisms
64 underlying these associations are often unclear and could perhaps be elucidated through the
65 examination of CNVs.

66 The vast majority of microbiome research to date, however, typically studies the
67 microbiome through the prism of relative abundance of microbial species, with only a small
68 number of studies focusing on the functional genetic level. Some studies analyzed the genetic
69 repertoire of the microbiome²¹ by mapping metagenomic reads to a collection of microbial genes
70 (e.g. ^{26,27}). While useful, this approach is limited as it usually analyzes microbial genes
71 separately from the microbes in which they are expressed, overlooking their genomic context
72 and membership in species-specific microbial pathways. Taxonomy-aware methods such as
73 FishTaco²⁸ and HUMAnN2²⁹, may supply information on microbial membership of genes, but is

74 limited in resolution with regards to within-species variation. Recently, Greenblum et al.¹⁵ have
75 performed a systematic characterization of intra-species CNVs across the human microbiome.
76 Both approaches, however, are limited by the scope of the annotation database used (KEGG²⁶
77 in the latter case¹⁵), and in any case do not account for co-variation of genes encoded in the
78 same genomic region. Such co-variation is important as it encodes information such as operon
79 membership, gene regulation, proximal RNA interference and susceptibility for horizontal
80 transfer that are only evident when analyzing genes in their immediate genomic context.

81 In this study, we focused on sub-genomic regions in the human microbiome that vary
82 across different hosts. We aimed to detect segments of varying lengths, potentially containing
83 multiple genes, that are deleted from certain bacteria in some individuals or present in a variable
84 number of copies in others. We term this phenomenon “sub-genomic variation” to differentiate it
85 from CNVs at the level of specific genes without genomic context (such as analyzed by
86 Greenblum et al.¹⁵).

87 One major difficulty in observing genes in their genomic context stems from the
88 challenge in correctly assigning metagenomic reads that originate from regions that are similar
89 between different bacteria. As many sequences are homologous between members of the same
90 taxonomic clade and others are potentially horizontally transferred between clades¹⁴, it is often
91 challenging to discern regions of high copy number within a genome from regions that are
92 present in multiple members of the metagenome. To overcome these issues, we devised an
93 Iterative Coverage-based Read Assignment (ICRA) algorithm that resolves ambiguous read
94 assignments using information on relative abundances of bacterial members of the microbiome,
95 sequencing-coverage across their genomes, and sequencing and alignment qualities. We show
96 that our algorithm correctly assigns reads in complex metagenomic settings.

97 We utilize our improved read assignment to develop a novel algorithm, SGV-Finder,
98 allowing us to detect 7479 microbial SGVs in 56 species from 7 microbial phyla in 887 human
99 microbiome samples^{20,30}, demonstrating that SGVs are widely prevalent in the human

100 microbiome. We show that these SGVs have distinct genetic functions, are associated with
101 bacterial growth rates, and are stable within the same person even over long periods of time,
102 altogether implicating SGVs as drivers of adaptation of a microbiome to a specific host
103 environment. We demonstrate the potential importance of SGVs to the human host by showing
104 124 cases in which SGVs are significantly associated with multiple disease risk factors. We
105 replicate our analysis in the Dutch Lifelines DEEP cohort^{31,32} and show that SGV positions
106 replicate in 76% of bacteria present in both cohorts, and that 40 associations with risk factors
107 also replicate, altogether suggesting that some genomic structural variability is shared between
108 distinct population, while some is population specific. We further demonstrate that examining
109 gene clusters in variable regions can reveal potential mechanisms of action, as in the case of an
110 *A. hadrus* region associated with multiple risk factors and whose genes code for a microbial
111 pathway which metabolizes sugar-alcohols to butyrate, a short-chain fatty acid (SCFA)
112 renowned for its advantageous effects on the human host³³⁻³⁵. Overall, we show that SGVs
113 represent a nascent layer of information in the human microbiome that is likely to be of high
114 relevance to human health.

115

116 **Results**

117

118 **Accurate metagenomic read assignment using the ICRA algorithm**

119

120 To accurately detect SGVs in the microbiome we sought to obtain a correct assignment of
121 metagenomic reads to their sequence of origin. Attaining such accurate assignment is
122 challenging due to the large number of genomic sequences that are shared across different
123 microbiome members. Here, we analyzed data collected on 887 healthy subjects which includes
124 microbiome profiling alongside detailed blood glucose measurements over the duration of a
125 week, anthropometric measurements, blood tests, and medical questionnaires^{20,30} (Methods). In

126 these 887 samples, over 15% of the metagenomic reads were assigned ambiguously to multiple
127 references upon mapping to a reference genome database of 3953 bacterial genomes³⁶ (Fig.
128 S1A, Methods).

129 To address this problem, we devised an Iterative Coverage-based Read Assignment
130 (ICRA) algorithm (Fig. 1A, Methods). In its first step, ICRA uses read assignments and mapping
131 qualities to calculate the sequencing coverage depth along microbial entities (e.g., bacterial
132 genomes or genes), and then uses this sequencing coverage to estimate microbial relative
133 abundances, while demanding sufficient coverage over entities that are to be considered
134 present in a sample (Methods). In the next step, ICRA reassigns reads using the updated
135 relative abundances, and repeats the process to convergence. The use of sequencing coverage
136 makes our method robust to genomic regions with extremely high or low coverage that may
137 arise from misassemblies, homology to other microbes, or phage activation. Such regions could
138 otherwise bias the estimated relative abundances, potentially even assigning abundances to
139 genomic entities that are not present in the sample, but contain a region homologous to other
140 entities present in reference databases.

141 To test the performance of ICRA, we validated the two key components of the algorithm:
142 its ability to resolve ambiguous read assignments, and the accuracy of the relative abundances
143 that it assigns to each bacterial species. To this end, we analyzed the assignment of reads from
144 simulated metagenomes provided by the CAMI challenge dataset along with their correct read
145 assignments³⁷. The CAMI dataset contains three sets of samples ranging from 30 to 450
146 genomes that account for varying microbiome complexities. We mapped each of these samples
147 to a reference of 482 bacteria derived from this dataset and compared the fraction of
148 metagenomic reads incorrectly or ambiguously assigned to reference genomes between a
149 baseline setting (uncorrected read assignment; Methods), the output of our algorithm, and two
150 state-of-the-art tools - Kraken³⁸ and MetaPhyler³⁹. Notably, we found that ICRA outperforms the

151 alternatives in assigning reads to reference genomes in both the species and sub-species
152 taxonomic levels in all complexity levels available from CAMI ($p < 0.01$; Fig. 1B, S1B,C)

153 As relative abundances are utilized by ICRA for the resolution of ambiguous read
154 assignments, we further validated that ICRA-derived relative abundances are comparable to
155 those derived from state-of-the-art tools created and optimized for this task. We therefore
156 compared microbial relative abundances produced by ICRA, to those derived from the popular
157 tools MetaPhlAn2⁴⁰, which uses marker genes to estimate abundances, and Bracken⁴¹, which
158 performs Bayesian reestimation of abundances derived with Kraken³⁸. To this end, and to best
159 simulate the genomic phenomena of bacteria growing naturally (rather than sampled *in silico*),
160 we obtained seven different bacterial strains, grew them to stationary phase, and extracted and
161 sequenced DNA from each strain separately (Methods). We then created 100 samples *in silico*
162 by randomly mixing reads sequenced from each of the seven strains at different relative
163 abundances, and applied MetaPhlAn2, Bracken and ICRA to these samples (Methods). We
164 found that while the Bray-Curtis dissimilarities between the relative abundances estimated by
165 these tools and the true relative abundances were lowest in Bracken (Fig. 1C, inset), followed
166 by ICRA and MetaPhlAn2, the abundances estimated by all three tools were comparable and
167 highly correlated with the true abundances ($R^2 > 0.93$ for each microbe across all samples,
168 $p < 10^{-10}$; Fig. 1C, S2).

169

170 **Sub-genomic variation is highly prevalent in the human microbiome**

171

172 We next sought to systematically characterize the landscape of sub-genomic variation across
173 the healthy human microbiome. To this end, we developed SGV-Finder, which we ran on ICRA-
174 corrected read assignments of 887 metagenomic samples^{20,30} to a reference database of 3953
175 representative microbial genomes derived from progenomes³⁶ (Methods). SGV-Finder analyzes
176 coverage-depth across all microbial genomes in all samples by dividing each genome to 1000

177 basepair bins and counting the number of reads mapped to each bin. To ensure proper
178 statistical support for copy number analyses, we discard genomes in samples whose median
179 bin coverage is lower than 10 reads (corresponding to a genome coverage of 1x, with ten 100bp
180 reads in each 1kbp bin; Methods), and microbial genomes present in less than 75 subjects. The
181 coverage depth of each genome in a given sample is then standardized by subtracting the
182 mean sample coverage and dividing by its standard deviation (Methods).

183 For detecting SGVs, we further differentiate between two SGV types. Deletion-SGVs are
184 sub-genomic areas that are deleted in enough subjects yet are present in others, and are
185 detected by searching for bins that are deleted in 25-75% of samples, with the read coverage
186 cutoff for deleted bins selected according to the distribution of read coverages (Methods).
187 Variable-SGVs are sub-genomic areas which have highly variable coverage across samples,
188 and are detected by fitting a beta-prime distribution on the standardized coverage of all samples
189 in a single bin, for bins that are not deleted in more than 5% of samples, and selecting bins with
190 abundance higher than 95% of values in the fitted distribution. In both variable- and deletion-
191 SGVs, detected bins are subsequently united based on cooccurrence (deletion-SGVs) or
192 correlation (variable-SGVs) (Methods). An online metagenome explorer for all SGVs and the
193 genes they encompass is available at <http://genie.weizmann.ac.il/SGV/> (Fig. S3).

194 Overall, we detected 2423 variable-SGVs and 5056 deletion-SGVs in 56 bacteria found
195 with sufficient coverage in at least 75 out of 887 samples (Fig. 2A). Sub-genomic variability was
196 detected in all 6 bacterial phyla and one archaeal phylum, with the number of variable or
197 deletion SGVs ranging from 5 to 241 SGVs per species in average sizes ranging between 1.4
198 and 18.6 kbp per species. Variable-SGVs make up between 0.3% and 8.4% of the microbial
199 genome while deletion SGVs exist in 5.0% to 26.9% of the genome (Fig. 2A). This apparent
200 disparity in size may suggest inherent differences in the formation of the two types of SGVs. Out
201 of 887 samples, 769 carried deletion- and variable-SGVs for *Blautia wexlerae*, 727 subjects had
202 104 deletion-SGVs and 33 variable-SGVs in *A. hadrus*, and 668 carried deletion- and variable-

203 SGVs for *Bacteroides uniformis*. Notably, we detected SGVs in all microbial strains that had
204 sufficient coverage, and in every subject analyzed, demonstrating the ubiquity of such
205 variations.

206

207 **SGV is prevalent across distinct populations and continents**

208

209 To test the universality of these regions and reinforce their biological relevance, we applied
210 ICRA and SGV-Finder independently to 1020 out of 1135 samples from the Dutch Lifelines
211 DEEP cohort^{31,32} which had sufficient sequencing depth (Methods). We found that in 47 out of
212 56 bacteria present in both cohorts, an average of 72.9% of variable-SGVs (0% to 99.1%) and
213 78.3% of deletion-SGVs (35.3% to 94.5%) overlapped with SGVs found in our cohort (one-sided
214 hypergeometric $p < 10^{-10}$; Fig. 2B,C). Notably, for 75% of microbes, more than 70% of the regions
215 were replicated despite the different populations examined with different genetic background,
216 cultural setting, and dietary preferences (Fig. 2C).

217 Some bacteria, such as *Ruminococcus bicirculanus*, showed very low concordance
218 between the two cohorts (27% overlap over 10 variable-SGV regions totalling 23kbp; Fig. 2B,C),
219 suggestive of geographical confinement of the variability, or a strong influence of population-
220 specific environmental factors. Conversely, other bacteria, such as *Parabacteroides merdae*,
221 showed high concordance (95% of 46 variable-SGVs totalling 281 kbp; Fig. 2B,C). Given the
222 different methods, centers, and staff involved in assembling the two cohorts, the replication of
223 the variable regions suggest that the variability detected here is not artifact but rather a
224 widespread phenomena in the gut microbiome across distinct geographical regions.

225

226 **SGVs are person specific and are shared with habitat**

227

228 We next examined the variability of SGVs across people by correlating the abundance of
229 variable- and deletion-SGVs between different subjects. We found that different individuals
230 mostly have different SGVs, with a median correlation of 0.02 and 0 for variable- and deletion-
231 SGVs, respectively (Fig. 2D,E). In contrast, SGVs were highly stable within the same individuals
232 even over time periods exceeding one year, with median within-person correlations of 0.89 and
233 0.66 for variable- and deletion-SGVs, respectively (Spearman correlation $p < 10^{-20}$ for both; Fig.
234 2D,E; Methods).

235 To estimate the effect of the environment and host genetics on SGVs, we analyzed data
236 from cohabiting individuals and for pairs of parents-children / siblings who do not live together⁴²
237 (Methods). We found that cohabiting individuals and parent-children / sibling pairs share both
238 deletion- and variable-SGVs to a significantly higher degree as compared to two randomly
239 chosen subjects from our cohort (average Spearman ρ of 0.45 and 0.16 for variable- and
240 deletion-SGVs, respectively; $p < 10^{-10}$ for both; Fig. 2D,E). Interestingly, siblings / parents-
241 children have a significantly less similar SGV profile in their microbiome as compared to
242 cohabiting subjects ($p < 0.001$ for both variable- and deletion-SGVs, Fig. 2D,E). This result is
243 conservative, as such similarity in the SGV profiles of genetically-related individuals cannot be
244 efficiently decoupled from confounders such as traditional food preferences or instances in
245 which these individuals share meals or experiences that may affect their microbiome as part of
246 their family get-togethers. These results replicate and strengthen our previous findings⁴²
247 showing that environment dominate over genetics in determining microbiome composition.

248

249 **Microbiome SGVs are potentially involved in microbial adaptation and function**

250

251 We sought to systematically characterize the functional landscape of SGV regions by examining
252 genetic functions that are enriched or depleted from SGVs. We annotated gene function across

253 variable- and deletion-SGVs, as well as in regions of microbial genomes that were covered
254 consistently in at least 98% samples that contained the bacteria (hereinafter termed 'conserved'
255 regions; Methods). We then performed enrichment analysis to seek for KEGG modules that
256 were over- and under-represented in these regions (Methods). Using the KEGG BRITE
257 hierarchy, we found that modules categorized into 'housekeeping' functions such as nucleotide
258 and amino acid metabolism or carbohydrate and lipid metabolism were significantly depleted
259 from variable- ($p < 10^{-5}$ for both groups; Fig. 2F; Table S1) and deletion- ($p < 10^{-5}$; Fig. 2G; Table
260 S1) SGVs and significantly enriched in conserved regions ($p < 10^{-5}$; Fig. 2H; Table S1).
261 Conversely, modules classified as ABC-2 type- and other transport systems were significantly
262 enriched in SGVs ($p < 10^{-5}$), possibly driven by the KEGG module pertaining to putative ABC
263 transporters ($p < 10^{-5}$; Fig. 2F). In addition, SGVs were enriched with the type-IV secretion
264 system (T4SS) KEGG module ($p < 10^{-5}$; Fig. 2F,G) suggesting that bacterial conjugation
265 systems, to which the T4SS is related, are strong drivers of variability. These systems were
266 strongly depleted from conserved regions ($p < 10^{-5}$; Fig. 2H) suggesting that they are much more
267 prevalent in the accessory genome compared to the core genome, and once more implicating
268 SGVs as tools of adaptation and speciation.

269 SGVs were additionally enriched with genes to which no function was assigned by
270 KEGG ($p < 10^{-5}$; Fig. 2F,G marked by a red star). To overcome this obstacle, we performed
271 enrichment analysis on word categories from the Ensembl functional annotation⁴³ of 167,389
272 genes in the 56 bacteria analyzed (Methods). Bacteriophage- and plasmid-related genes, genes
273 associated with transposable elements, and genes encoding other horizontal gene transfer
274 (HGT) mechanisms were strongly enriched in variable- (FDR-corrected $q < 10^{-4}$) and deletion-
275 SGVs ($q < 10^{-4}$) and strongly depleted from conserved regions ($q < 10^{-4}$), suggesting an important
276 role for these mechanisms in the formation of these regions. Analysis of Pfam⁴⁴ motifs
277 pertaining to HGT mechanisms (Methods) corroborated this finding and showed an enrichment
278 of phage-, prophage-, transposon and conjugated-transposon-related motifs in variable- and

279 deletion-SGVs and their depletion from conserved regions ($q < 10^{-4}$). In addition, variable-SGVs
280 were enriched with antibiotic-producing genes ($q < 0.005$) and deletion-SGVs were enriched with
281 CRISPR-associated genes ($q < 0.05$) suggesting that these regions function as attainable
282 microbial tools for interacting with their environment. This analysis also demonstrates how SGV-
283 Finder, which operates directly at the genomic level, can accommodate analyses with multiple
284 annotation datasets.

285 To further characterize the potential contribution of SGVs to microbial niche adaptation,
286 we searched for regions that are associated with fitness of their harboring microbe. As a proxy
287 for fitness, we calculated bacterial growth rates of 21 bacterial strains with sufficient coverage
288 and available complete genomes using a method we previously developed that estimates
289 growth through differences in DNA copy number at the origins and terminus locations created
290 during DNA replication⁴⁵. We found 44 highly significant associations (surpassing Bonferroni
291 correction cutoff of $p < 3 \times 10^{-5}$; Fig. 2I; Table S2) of these growth rates with deletion-SGVs within
292 the same bacteria (Methods). These significant associations span a total of 8 distinct bacteria,
293 suggesting that certain SGVs may be important for bacterial adaptation and fitness.

294 To better probe the mechanisms potentially underlying this adaptation, we systematically
295 examined the genetic content of the deletion-SGVs that were significantly associated with
296 growth, and found a similar pattern to that seen when analyzing all SGVs, with a depletion of
297 housekeeping functions and enrichment for genes involved with CRISPR-, transposon- and
298 HGT-associated genes ($q < 0.05$; gene categories based analysis; Methods), as well as a
299 significant enrichment for genes with unknown functions ($p < 10^{-5}$, Fig. S4).

300 We further examined two such regions, which were significantly positively and negatively
301 associated ($p < 10^{-10}$ for both) with the growth of the same harboring species (*Eubacterium*
302 *eligens*; Fig. S5A-D). Notably, the SGV whose presence is negatively associated with the
303 growth dynamics of the microbial host (Fig. S5A,B) contain genes for flagellin, flagellar hook-
304 associated protein and lipopolysaccharide (LPS) choline phosphotransferase among a few

305 metabolic genes and response regulators (Table S3). Flagellin and the flagellar hook protein
306 were shown to elicit strong immune responses in mammals^{46,47}, possibly inhibiting bacterial
307 growth. LPS choline phosphotransferase attaches choline phosphate to the bacterial LPS
308 molecule, which was shown to increase C-reactive protein-mediated innate immune clearing⁴⁸,
309 again suggesting possible inhibition of microbial growth. Thus, increased growth rates in
310 bacteria missing these subgenomic regions may point to loss-of-function adaptation of these
311 bacteria to the host gut and its immune system. In contrast, the SGVs whose presence was
312 positively associated with their microbial host growth dynamics (Fig. S5C,D) contained mostly
313 hypothetical coding genes, but also a gene for antibiotic transport system ATP-binding protein,
314 whose presence could have a selective advantage in the human host by conferring resistance
315 to antibiotics⁴⁹ (Table S3). These results demonstrate the ability of our methodology to suggest
316 underlying mechanisms using the genomic context of SGVs.

317 Overall, our results show that SGVs associate with common mechanisms of conjugation,
318 transposition and phage lysogeny, and may thus be powerful tools of niche adaptation. The
319 acquisition of bulk genetic material not present in a microbial genome, and changes in copy
320 number of regions that are, may be much stronger drivers of adaptation than rarely occurring
321 point mutations. Microbial evolution in densely populated ecosystems such as the human
322 microbiome may thus be driven strongly by SGVs, which allow incorporation of functional
323 genetic material conferring higher fitness, and affecting both microbes and host.

324

325 **Microbiome subgenomic variation is associated with host disease risk factors**

326

327 To explore the potential relevance of microbiome SGVs to human health, we used data
328 collected on 887 subjects which includes microbiome profiling alongside detailed blood glucose
329 measurements over the duration of a week, anthropometric measurements, blood tests, and
330 medical questionnaires^{20,30}. We associated the abundance of variable-SGVs and the presence

331 or absence of deletion-SGVs with multiple metrics of health and metabolic risk factors: mean
332 arterial blood pressure (MAP); total and HDL cholesterol; waist circumference; body weight;
333 body mass index (BMI); median glucose levels over the measured week; percent glycosylated
334 hemoglobin (HbA1C%); and age. We found 81 (Fig. 3A, S6) and 43 (Fig. 3B) significant
335 associations at a false discovery rate (FDR)⁵⁰ of 0.1 for variable- and deletion-SGVs,
336 respectively, potentially demonstrating the importance of SGVs not only to the microbe, but also
337 to the host.

338 Several of the associations of risk factors and SGVs found in this study are in line with
339 the associations of the harboring microbe. For example, we found five deletion-SGVs in *A.*
340 *hadrus* to be associated with lower BMI, body weight and waist circumference, and with higher
341 HDL cholesterol levels (Fig. 3B), and we indeed found this bacteria to be negatively correlated
342 with body weight ($p < 10^{-5}$), waist circumference ($p < 10^{-5}$), median blood glucose levels ($p < 10^{-4}$)
343 and BMI ($p < 0.005$) and positively correlated with HDL cholesterol levels ($p < 10^{-7}$). Additionally,
344 this bacteria was previously shown to increase in abundance following a very low calorie diet⁵¹.
345 Despite being both correlated with similar risk factors, the association of the highlighted SGV
346 with risk factors allows us to pinpoint specific regions and mechanism that may underlie the
347 association.

348 In some cases, we potentially expose novel associations between the microbiome and
349 disease as some associations between host phenotypes and SGVs do not take the same
350 direction as the associations of the same phenotypes with the abundances of the harboring
351 bacteria. For example, three variable-SGVs in *Ruminococcus torques* were negatively
352 associated with multiple risk factors for the metabolic syndrome (Fig. 3A) but we found *R.*
353 *torques* abundance to be positively associated with body weight ($p < 10^{-3}$) and BMI ($p < 0.05$), and
354 it was also positively associated with the metabolic syndrome in a different cohort⁵². Similarly,
355 several variable-SGVs in *Eubacterium rectale* were positively associated with age (Fig. 3A),
356 while the relative abundances of *E. rectale* were negatively associated with it ($p < 10^{-6}$). A 2-kbp

357 deletion-SGV in *Faecalibacterium cf. prausnitzii* KLE1255 was positively associated with the
358 weekly median glucose level (Fig. 3B), and even though *F. prausnitzii* was not significantly
359 associated with median blood glucose levels in our cohort, two independent studies found it to
360 be negatively associated with type II diabetes mellitus, a disease for which blood glucose levels
361 are a major risk factor^{21,53}. These seemingly paradoxical associations between SGVs and
362 disease-risk factors further suggest that SGVs represent a different layer of information
363 compared to the taxonomic level, one which may assist in obtaining mechanistic insights into
364 the etiology of gut microbiota-associated metabolic disease.

365

366 **Disease risk-associated SGVs replicate in the Dutch Lifelines DEEP cohort**

367

368 To test the replicability of these associations, we ran ICRA on read assignments from the
369 Lifelines DEEP cohort, and used the corrected assignments to calculate the coverage and
370 presence/absence of variable- and deletion-SGVs as defined from the 887-person cohort. We
371 then calculated the association of these regions with similar host disease risk factors measured
372 in the Lifelines DEEP cohort, and compared those to the associations with metabolic risk factors
373 found in our cohort (Methods). Notably, despite presumed inter-cohort differences in genetics,
374 dietary preferences and lifestyles, potentially also leading to differences in the etiology of
375 metabolic disease between the two cohorts, more than a third (40 out of 117) of the
376 associations found in our cohort in microbes also present in the Lifelines cohort were replicated,
377 while only 4 out of the remaining 77 were significantly associated in the opposite direction (Fig.
378 3A,B; Fig. S6).

379

380

381 **Disease risk-associated SGVs facilitate an investigation of putative mechanisms**

382

383 As in the case of bacterial adaptation, examining the genetic content of SGVs facilitated a
384 potentially mechanistic view into the observed phenomena, and we therefore next looked into
385 the functions encoded in disease risk-associated SGVs. While many SGVs harbor genes that
386 are of unknown function, we did observe several intriguing functions coded in SGVs associated
387 with disease risk factors. For example, the existence of a 11-kbp deletion-SGV from *E. rectale* is
388 associated with higher HbA1C% ($p < 10^{-4}$; total 630 subjects, 377 retaining; Fig. 3C). A close
389 examination of this region reveals a class 1 CRISPR-Cas system (Fig. 3D). While it is unclear
390 how a CRISPR system could be directly related to host disease risk factor, we note the
391 existence of additional three genes of unknown function in this region. Interestingly, subjects
392 with *E. rectale* harboring this region had a higher abundance of the microbe (Mann-Whitney U
393 $p < 0.02$), which we had previously shown to increase in abundance following a diet designed to
394 induce high postprandial glucose responses²⁰. A 6-kbp variable-SGV from *R. torques* is
395 inversely associated with weekly median glucose levels ($R = -0.237$, $p < 10^{-5}$; Fig. 3E) and features
396 several genes encoding phage-associated proteins and additional genes of unknown function,
397 suggesting that this SGV is a prophage, and that it may carry additional functionality (Fig. 3F).
398 These genes of unknown function are therefore putatively related to host glucose metabolism,
399 demonstrating the utility of our methods for generating mechanistic hypotheses.

400 Other intriguing examples for putative mechanisms include a 4-kb deletion-SGV in *A.*
401 *hadrus* that is significantly associated with lower BMI (median lower by 1.15 kg/m² in subjects
402 retaining the region; $p < 10^{-4}$; total n=681, 405 retaining; Fig. S7A) and body weight (median
403 lower by 3.5 kg; $p < 10^{-4}$). This SGV contains genes coding for the enzymes ADC synthase (EC
404 2.6.1.85) and 4-amino-4-deoxychorismate lyase (EC 4.1.3.38), both instrumental in folate
405 biosynthesis in *A. hadrus* (Fig. S7B, C). An 18-kb deletion-SGV in *Roseburia intestinalis* that is
406 significantly associated with total cholesterol (median lower by 12.5mmHg for subjects retaining

407 the region; $p < 10^{-4}$; $n = 262$, 68 retaining; Fig. S7D) contained multiple beta- and other
408 glucosidases (Fig. S7E), potentially suggesting microbial adaptation to a fiber-rich host diet. An
409 8-kb deletion-SGV in *Coprococcus comes* which is significantly associated with BMI (median
410 higher by 2.4 kg/m² for subjects retaining this region; $n = 450$; 292 retaining; $p < 10^{-5}$; Fig. S7F)
411 and body weight (median higher by 5 kg; $p < 10^{-4}$) contains several ABC transporters with
412 undetermined substrates of possible future interest (Fig. S7G).

413 Notably, all of the above regions of interest were also detected as SGVs in the Lifelines
414 DEEP cohort (Fig. S8) and replicate the patterns of deletion or variation across the region that
415 were detected in our cohort.

416

417 **Carbohydrate metabolism and SCFA biosynthesis gene clusters encoded in a disease** 418 **risk-associated region**

419

420 As one particularly intriguing example, a 31-kbp deletion-SGV in *A. hadrus* was significantly
421 associated with lower body weight (median 6kg lower for subjects retaining the region; $p < 10^{-6}$;
422 $n = 681$, 468 retaining; Fig. 4A), waist circumference (median lower by 4 cm; $p < 10^{-4}$; Fig. S9A)
423 BMI (median lower by 1.17 kg/m²; $p < 0.001$; Fig. S9B), and higher HDL cholesterol (median
424 higher by 5.7 mg/dL; $p < 10^{-4}$; Fig. S9C), and was well annotated, allowing us to speculate about
425 its possible role in the microbiome, and demonstrating the potential of SGV-finder detected
426 regions to expose potential underlying mechanisms.

427 This genomic region encodes two full metabolic modules, seven sugar transporters and
428 two transcriptional regulators, among several unrelated genes (Fig. 4B). Of the two metabolic
429 modules, one performs inositol catabolism⁵⁴ metabolizing myo-inositol or D-chiro inositol to (a)
430 glycerone phosphate, a precursor for glyceraldehyde-3-phosphate, a constituent of the
431 Embden–Meyerhof–Parnas glycolysis pathway²⁶; and (b) 3-oxopropanoate, a precursor for
432 acetyl-CoA. The second metabolic module encoded in this SGV metabolizes 3-

433 hydroxybutanoyl-CoA to butyrate, a short-chain fatty acid (SCFA), while oxidizing an electron-
434 transferring flavoprotein encoded in the same SGV. The two pathways are connected through a
435 series of reactions encoded elsewhere in the *A. hadrus* genome (Fig. 4C, Table S4). Of the
436 sugar transporters, one is specific to the sugar alcohol sorbitol and six were not assigned a
437 specific target.

438 Combining the information regarding the two metabolic modules and the glucose
439 transporters in this SGV, we hypothesize that this region is unifunctional, providing the
440 bacterium with the capability to ferment sugar alcohol such as inositol to SCFAs in an
441 energetically-favorable procedure. The combined effect of the two metabolic pathways on the
442 energy metabolism of *A. hadrus* is positive, earning a net gain of 2 ATP- and 2 NADH-
443 equivalent molecules, where the myo-inositol catabolism module combined with glycolysis and
444 acetyl-CoA synthesis have a positive energetic effect and the butyrate synthesis module
445 consumes energy for butyrate production.

446 This 31-kbp deletion-SGV in *A. hadrus* was replicated with the Dutch cohort (Fig. S8),
447 and so were several of its association with host phenotypes: Dutch individuals harboring the
448 region exhibiting lower BMI (median lower by 0.9kg/m² for individuals retaining the region;
449 p<0.005; Fig. S9D), body weight (median lower by 4kg.; n=797, 547 retaining; p<0.01), and
450 waist-to-hip ratio (median lower by 0.017; p<0.001) potentially pointing to a generalized
451 mechanistic association between SGV and disease-risk.

452 In order to study the metabolic context of this adaptation in a broader ecological context,
453 we applied mimosa⁵⁵ to obtain the metabolic potential of the metagenomes of different subjects
454 and compared the differences between the community metabolic potential (CMP) of compounds
455 in subjects for whom the SGV is deleted and for subjects in which it is retained. We found that
456 free (unphosphorylated) sorbose, mannitol, galactitol and sorbitol are decreased in individuals
457 retaining the region (FDR adjusted two-sided Mann-Whitney *U* q<10⁻⁴, q<0.01, q<0.05 and
458 q<0.1, respectively; Table S5), whereas sorbose-1-phosphate, mannitol-1-phosphate and

459 sorbitol-6-phosphate are increased ($q < 10^{-4}$, $q < 0.01$ and $q < 0.05$, respectively; Table S5),
460 altogether demonstrating an association between adaptation in a specific bacteria to the
461 metabolic state of the microbiome, in the context of metabolic disease risk. As phosphorylation
462 is used in the phosphotransferase system to prevent sugar diffusion out of the cell, these
463 predictions support our observed increase in sugar-alcohol transport. Thus, we hypothesize that
464 the contribution of this SGV to the overall metabolic function of the microbiome is such that it
465 increases SCFA production from sugars and consequently exerts beneficial effects on the host.

466

467 **Discussion**

468

469 In this work we uncover a new facet of host-microbiome interactions in the context of health and
470 risk of disease. We present ICRA, a metagenomic read assignment algorithm, which we
471 validate by showing superior read-assignment and comparable bacterial abundance estimation
472 with respect to state-of-the-art algorithms. We also present SGV-Finder, a genomic coverage-
473 based algorithm for the detection of SGVs across metagenomic samples. Using this algorithm,
474 we show that SGVs are highly abundant in the human microbiome, and are largely conserved
475 across cohorts that differ in their genetic, cultural and dietary backgrounds. SGVs are host-
476 specific, conserved in the same individual over time and are more conserved in cohabiting
477 vs. genetically-related individuals. We found that SGVs harbor genes of distinct functions, and
478 are associated with bacterial growth rates, indicating a potential utility in bacterial adaptation.
479 Finally, we found that SGVs are associated with numerous host disease risk-factors, many of
480 which replicated across two independent cohorts, and that they facilitate exploration of genes
481 varying together, exposing a new layer of putative mechanistic information regarding host-
482 microbiome interactions, which we highlight by the discovery of a potentially butyrate-producing
483 SGV in *A. hadrus*.

484 To our knowledge, ICRA is the first metagenomic read assignment algorithm to
485 introduce the demand that for a genetic element, whether bacteria, genomic region, or gene, to
486 be considered present in the sample, its genomic sequence should be sufficiently covered by
487 metagenomic reads. This precondition increases robustness to shared genomic regions,
488 assembly errors, and phage activation. We note that a challenging problem which ICRA does
489 not address is the lack of accurate reference genomes for many of the microbial members of the
490 gut microbiome. De novo long-read approaches to generate reference genomes from
491 metagenomes such as Molecu⁵⁶ and the 10x platform⁵⁷ could prove useful in this context.
492 Combined with ICRA and SGV-Finder these approaches would successfully delineate additional
493 interpersonal differences in sub-genomic regions of the microbiome.

494 Using SGV-Finder, we show that SGVs are highly abundant in the human microbiome,
495 with variable regions present in all 56 microbes from 7 different microbial phyla which had
496 sufficient coverage, 46 of which replicate to a high degree in an independent cohort. Following a
497 functional analysis of genes in those regions, we hypothesize that the main forces driving SGVs
498 are bacteriophage infections and microbial mechanisms of conjugation and transposable
499 elements, as evident from the high abundance of genes performing such functions in SGV
500 regions. However, many genes found in SGVs, such as antibiotic biosynthesis genes, can
501 possibly be characterized as passengers to this process of transposition and may have
502 important roles in the adaptation of microbes to their ecological niche and in communication with
503 the host. We show many SGVs are strongly linked to microbial growth, a proxy for fitness,
504 demonstrating the potential functional importance of SGVs in their harboring microbe.

505 Our results show that SGVs also associate with host disease risk. We found more than
506 120 significant associations between SGVs and multiple metrics of metabolic disease,
507 highlighting their potential relevance to host health. Notably, more than one third of the
508 associations testable in an independent cohort were replicated, demonstrating the conserved
509 association of these SGVs to disease risk. Many of these regions demonstrate associations with

510 host health that are in opposite direction to the associations found between their harboring
511 microbe and disease risk, indicating that this is a complimentary layer of information to that of
512 taxonomical abundances.

513 We have closely examined these regions and the genes that they harbor, and
514 demonstrated the utility of such examination with several SGVs whose genes were well
515 annotated, including a 31-kbp SGV that was strongly associated with lower metabolic risk
516 across multiple biomarkers and which we also found to encode a bacterial pathway pertaining to
517 the transport and fermentation of sugar alcohols to the short chain fatty acid butyrate. SCFAs,
518 and specifically butyrate, have been previously shown to nourish host intestinal cells^{58,59} and
519 mitigate inflammatory disease⁶⁰. In mice, SCFAs were shown to improve insulin sensitivity and
520 increase energy expenditure⁶¹, suggesting that the inclusion of this SGV in the bacterial genome
521 and thereby the potential boosting of SCFA production may be advantageous for both the
522 bacteria and host metabolism. We hypothesize that by possessing this SGV, bacteria
523 demonstrate increased symbiosis with the host, as fermenting sugar alcohols to butyrate
524 benefits the microbe by producing additional energy and benefits the host with the
525 advantageous effects of intestinal butyrate.

526 Despite the visible links between this SGV and host metabolism, and between this SGV
527 and bacterial metabolism, we do not know whether the SGV leads to the observed lean
528 phenotype or whether the diet, lifestyle and other factors in the host lead to the incorporation or
529 loss of this SGV. While further research is needed to fully understand the links between host
530 diet and lifestyle, the microbiome and metabolic disease, this SGV demonstrates the wealth of
531 mechanistic knowledge obtained through examining genes with variable copy number in their
532 genomic context and along with neighboring variable genes. This type of analysis, connecting
533 genomic variation with genetic function, could be instrumental for raising multiple mechanistic
534 hypotheses about the pathophysiological role of the microbiome. We therefore made our

535 algorithms available for the scientific community and developed an online metagenomic SGV
536 explorer that will enable further exploration (all available at <http://genie.weizmann.ac.il/SGV/>).

537 The current implementation of both ICRA and SGV-Finder depends on a genomic
538 reference dataset, which are typically sufficient for human microbiome analyses. Even so, we
539 note that this is a practical rather than a conceptual approach, as the algorithms are capable of
540 running on any type of database of genetic elements. Future work could validate and use these
541 methods following metagenome assembly, ORF prediction and functional prediction stages,
542 which would allow their application to different host-associated environments and different
543 realms of microbiology and cellular biology, such as to soil or extreme microbiomes.

544 Our methodology is highly adaptable to any metagenomic scenario and could be used,
545 for example, to detect SGVs in the soil microbiome and associate them with the presence of
546 specific nutrients and metabolites to detect candidate biosynthetic gene clusters. Taken
547 together, our study exposes a new facet of the microbiome that brings us closer to
548 mechanistically understanding links between microbe and host.

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- 695

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711

712 **Author contributions**

713 T.K. and D.Z. conceived the project, designed the study, designed and conducted all analyses,
714 interpreted the results, and wrote the manuscript. T.K. and D.Z. equally contributed to this work
715 and are listed in random order. A.G. and N.B. developed methods. A.K., J.F., C.W. and A.Z.
716 performed the analyses of the Dutch cohort. M.L.-P and A.W. did experimental work on the 7
717 strains. A.W. designed the study. E.S. conceived and directed the project and analyses,
718 designed the analyses, interpreted the results and wrote the manuscript.

719

720

721

722 **Methods**

723

724 Reference database preprocessing

725 We downloaded the EMBL progenomes³⁶ 5306 representatives dataset and used dRep⁶² to
726 calculate distances between genomes. Next, we applied ward hierarchical clustering with a
727 Euclidean distance metric to the dRep distance matrix, calculated a dendrogram and retrieved
728 the cut tree at a height of 0.15 (corresponding to approximately 15% dissimilarity in genome
729 sequence) resulting in 3953 clusters. As a representative species for each cluster we chose the
730 genome with the minimal distance to all other genomes in the cluster. In clusters with only two
731 members, we chose one randomly. Database taxa and assembly accession numbers are listed
732 in Table S6.

733

734 Metagenomic samples - Israeli cohort

735 We obtained metagenomic samples from two studies^{20,30} (accession numbers ENA:
736 PRJEB11532, ENA: PRJEB17643). In the latter study³⁰, only baseline samples were used
737 (before the intervention took place).

738

739 Gut microbiome analysis

740 To prevent bias generated by analyzing single- and paired-end sequenced samples together,
741 we took the first end of all samples, and trimmed each read to a maximal length of 75bp (100bp
742 for Lifelines DEEP cohort). We filtered metagenomic reads containing Illumina adapters, filtered
743 low quality reads and trimmed low quality read edges. We detected host DNA by mapping with
744 GEM⁵⁰ to the Human genome with inclusive parameters, and removed those reads. We
745 randomly subsampled all samples to 10M reads, and removed samples with less than 10M
746 reads from subsequent analyses.

747 For MetaPhlan2 comparisons, we obtained relative abundances (RA) from metagenomic
748 sequencing via MetaPhlan2⁴⁰ with default parameters. For Kraken³⁸ comparisons, we built a
749 custom Kraken database using our preprocessed database and subsequently classified with
750 default parameters and generated a Kraken report. For Bracken⁴¹ abundance estimation, we
751 generated a Bracken-database file using bracken-build on the above Kraken database with a
752 kmer length of 31 and read length of 100bp and used it to estimate abundance using the
753 aforementioned Kraken report.

754

755 ICRA - Iterative Coverage-based Read Assignment algorithm

756 We devised an iterative read assignment algorithm which uses read assignments and
757 sequencing qualities to calculate the sequencing coverage depth along genomic elements (i.e.,
758 bacterial genomes or gene sequences) in the microbiome. Sequencing coverage is then used to
759 both qualitatively assess the presence or absence of each microbe by demanding a minimum
760 coverage across each genomic element, as well as to quantitatively estimate the relative
761 abundance of each microbe disregarding outlier genomic positions where extremely high or low
762 coverage exists. Microbial relative abundances are subsequently used to estimate read
763 assignments, repeating the process to convergence.

764 For a more formal description of our algorithm, let $i = 1, 2, \dots, R$ be the index of
765 metagenomic reads in a sample; let $j = 1, 2, \dots, G$ be the index of genomic elements in a
766 database of such elements; and $p(i, j)_k = p(i, j)_1, p(i, j)_2, \dots, p(i, j)_{N(i, j)}$ be all the possible
767 alignment positions for read i in genomic element j ($N(i, j)$ is the total number of possible
768 alignments of i to element j , in most cases only one) such that if metagenomic read i is assigned
769 to position $p(i, j)_k$, it spans an alignment from $p(i, j)_k$ to approximately $p(i, j)_k + \rho_i$, where ρ_i is
770 the length of read i .

771 Our goal is, therefore to find, for each i, j and k , $\lambda_{i,j,k}$, an indicator variable for the origin
772 of read i :

773 $\lambda_{i,j,k} = 1$ iff read i originated from genomic element j in position $p(i, j)_k$
774 To approximate $\lambda_{i,j,k}$, we calculate, for each read the probability $\delta_{i,j,k}$ that read i
775 originated from the genomic element j at position $p(i, j)_k$, as:

$$776 \quad \delta_{i,j,k} = \frac{\pi_j \theta_j q_{i,j,k}}{\sum_{l,m} \pi_l \theta_l q_{i,l,m}}$$

777 Where:

778 • $\pi_j = f(\{\delta_{i,j,k} \forall i, k\})$

779 π_j is the estimated relative abundance of the genomic element j . In the initial iteration of
780 the algorithm, π_j is calculated by counting all reads mapped to genomic element j and
781 then dividing the result by the total number of reads. Reads mapped to multiple genomic
782 elements are initially distributed according to quality of mapping (see q below).
783 Function f divides the genomic element j to bins of a size defined by the user (1kbp by
784 default), calculates bin coverage by summing all $\delta_{i,j,k}$ (from previous iteration) in each
785 genomic bin, and calculates π_j as the median of the $n\%$ most closely covered bins in the
786 genomic element, with n defined by the user. For the default n of 60, we calculate the
787 difference between the most covered bin and the least covered bin for every subset
788 spanning 60% of the bins, find the subset in which the difference is minimal, and take its
789 median coverage. This median is then multiplied by the number of reads to reach an
790 estimation of the true number of reads originating from the genomic element j . This
791 number is then divided by the total number of reads assigned to all genomic elements to
792 calculate π_j . π_j is then normalized by the length of the genomic element (or its harboring
793 microbe), but this could be turned off by the user.

794 • $\theta_j = \sum_{i,k} I_{i,j,k}$

795 Where $I_{i,j,k} = 1$ iff $\delta_{i,j,k} > \delta_{i,l,m} \forall l, m$

796 i.e., the sum of reads preferentially mapped to this genomic element. This parameter
797 facilitates faster convergence but results in reduced accuracy, and is suggested for use
798 in case of very large reference datasets. With default ICRA parameters, it will be set to 1
799 (and therefore ignored).

800 • $q_{i,j,k} = \prod_{pos=0}^{\rho} qual(pos)^{\mu(i,j,p(i,j)_k+pos)} (1 - qual(pos))^{1-\mu(i,j,p(i,j)_k+pos)}$

801 is the probability of a correct mapping, given the mismatches in the read and the
802 sequencing qualities. Where $qual(pos)$ is the probability of correct sequencing in position
803 pos calculated from fastq qualities and $\mu(i,j,p(i,j)_k + pos) = 1$ if there is a match
804 between nucleotide in position pos in read i to the one in position $p(i,j)_k+pos$ in genomic
805 element j and 0 otherwise.

806 • The term $\sum_{l,m} \pi_l \theta_l q_{i,l,m}$ is used to normalize $\delta_{i,j,k}$ such that the sum of all possible
807 assignments of read i equals 1, where l and m refer to all possible genomic elements
808 and positions thereof to which read i is mapped.

809 If $\delta_{i,j,k}$ is lower than a user-set parameter ϵ , with a default of 10^{-6} , this specific mapping is
810 removed from subsequent analysis thereby reducing noise typically originating by highly
811 homologous regions from in subsequent iterations.

812

813 CAMI dataset comparison

814 We downloaded all 180bp-spaced toy datasets for the 1st CAMI challenge³⁷ from the CAMI
815 challenge website (<https://data.cami-challenge.org/participate>). We created a database of all
816 taxonomic entities in CAMI using NCBI taxon IDs provided for all gold-standard abundances.
817 We indexed this database using GEM indexer⁶³ and mapped all metagenomic reads to the
818 indexed database using GEM mapper. In the baseline setting, read assignment was not
819 corrected using ICRA, and the assignment of reads that were mapped to more than one
820 genome was a uniform division between these genomes. In the ICRA-corrected setting, read
821 assignment was given by applying ICRA to GEM mapper output. For MetaPhyler³⁹ read
822 classification, we created a MetaPhyler classifier based on the same CAMI reference database

823 using the *buildMetaphyler.pl* command with a sequence length of 100bp and classified CAMI
 824 reads using the *runClassifier.pl* command with default parameters. For Kraken³⁸ comparison,
 825 we built a custom Kraken database based on the same CAMI reference database and ran
 826 Kraken as above. The four resulting assignment sets were compared to the gold standard
 827 provided by CAMI to derive correct assignment ratios.

828

829 Bacterial strain culture and sequencing

830 The following strains were obtained and grown in the following conditions:

Species	Strain ID	Growth condition – Medium	Growth condition - Temp	Growth to saturation
<i>Lactobacillus gasseri</i>	ATCC 33323	Lactobacillus MRS agar	37°C	24 hrs
<i>Enterococcus faecalis</i>	ATCC 29212	ATCC Medium 44	37°C	overnight
<i>Streptococcus cristatus</i>	ATCC 51100	ATCC Medium 44	37°C	<24 hrs
<i>Akkermansia muciniphila</i>	ATCC BAA-835, DSM 22959	DSM medium 104 + 0.05% mucin or ATCC medium 44	37°C	72 hrs
<i>Cellulomonas flavigena</i>	ATCC 482, DSM 20109	DSM 53 or ATCC Medium: 3 Nutrient Agar/Broth	30°C	72 hrs
<i>Brachybacterium faecium</i>	ATCC 43885, DSM 4810	DSM 92 or ATCC Medium: 3 Nutrient Agar/Broth	30°C	72 hrs
<i>Alistipes finegoldii</i>	DSM 17242	DSM medium 104 + vitamin solution (see medium 131) or 693	37°C	> 24 hrs

831

832 Strains were grown to stationary phase as listed in the table. DNA was extracted using
833 QIAgen DNAeasy Blood & Tissue kit (Cat# 69504) by the protocol using pretreatment of Gram-
834 positive or Negative bacteria following purification of total DNA from animal tissues.

835 Following that, 100 ng of DNA was sonicated using Covaris E220X and and Illumina
836 library was prepared for each strain as previously described⁶⁴. The seven strains were
837 sequenced to a minimum depth of 3M reads by a NextSeq® 500 machine with Illumina NS
838 500/550 High Output V2 75 cycle kit. Data was deposited to ENA, accession ENA:
839 PRJEB25194.

840

841 SGV detection - preprocessing

842 We mapped metagenomic reads to the reference database of 3953 representative microbial
843 genomes detailed above and corrected read assignments using ICRA. All scaffolds from each
844 microbial genome were concatenated and subsequently divided into 1 kbp bins. For each
845 genome in each microbial sample, we counted the number of reads mapped to each of the bins.
846 In the rare case in which ICRA produces a distribution of probabilities of different read
847 assignment for a specific read rather than a deterministic assignment, we determined the read
848 count that was added to each bin using the probability of assignment calculated by ICRA.
849 Microbes with a median coverage smaller than 10 reads per bin were discarded from
850 subsequent analyses. In addition, we removed microbes in which the median bin coverage
851 across samples was lower than one read for more than 30% of the bins.

852

853 Detection of deletion SGVs

854 We examined the coverage in each metagenomic bin across all samples to detect regions that
855 were deleted from some individuals and retained in others. To this end, for each microbe in
856 each sample, we calculated a histogram of coverage across all metagenomic bins. We then
857 searched for a trough, separating bins whose coverage is close to 0 from bins whose coverage

858 is close to the median across the microbe, which we previously demanded to be greater than 10
859 reads. The position of the trough separates the two modes of the distribution, between bins
860 which were deleted (number of reads per bin smaller than the trough position) and retained
861 (number of bins greater than the trough position). To mark a bin as a potential deletion-SGV, we
862 demanded that it be deleted in 25-75% of samples. We concatenated adjacent deletion-SGV
863 bins into stretches based on bin cooccurrence dissimilarity, defined as the proportion of samples
864 which are in disagreement on the deletion-state of the two bins being compared (wherein one
865 bin is deleted and one is retained for the same sample) out of all samples that harbor the
866 microbe. Bins were concatenated to an existing stretch if they had an average cooccurrence
867 dissimilarity lower than 0.25 with all the bins in the stretch, and that the newly created stretch is
868 deleted in 25-75% of samples. We then clustered deletion SGV stretches belonging to the same
869 microbe based on cooccurrence. First, we calculated a cooccurrence dissimilarity matrix for any
870 two bins within the microbe (calculated as 1 minus the cooccurrence metric defined above).
871 Next, using this bin-dissimilarity matrix we calculated a region dissimilarity matrix by calculating
872 the average distance between all bins of one region to all bins of the other region. We next
873 calculated linkage over the bin-dissimilarity matrix using the 'average' method of the
874 cluster.hierarchy.linkage function in scipy v1.1.0 and divided into clusters with maximal
875 cooccurrence dissimilarity of 0.25.

876

877 Detection of variable SGVs

878 For each microbe, we first removed all bins that were deleted in more than 95% of subjects. We
879 examined the coverage in each remaining metagenomic bin across all samples to detect
880 regions with variable coverage. To this end, we standardized the coverage across all non-
881 deleted bins of a single microbe in each sample by subtracting the mean coverage and dividing
882 by the standard deviation. Next, for each bin, we fit a beta-prime distribution over all samples
883 and marked bins whose value is in the top 5th percentile of the fit distribution as variable SGV.

884 We concatenated adjacent variable SGVs into stretches if their average correlation (Spearman)
885 with all bins in the stretch was higher than 0.75 and the resulting stretch was in the top 5th
886 percentile of the beta-prime fit distribution of the resulting bin size. We then clustered variable
887 SGV stretches similarly to deletion SGV stretches, with a dissimilarity metric calculated as $1 -$
888 $((\rho(u,v)+1)/2)$, where ρ is the Spearman correlation and u, v are the bin vectors being compared;
889 and threshold 0.125. This roughly corresponds to an average Spearman correlation threshold of
890 0.75.

891

892 Detection of conserved regions

893 For each microbe in each sample, we detected retained / deleted bins as above and defined
894 conserved regions to be stretches of bins that were deleted in less than 1% of samples.

895

896 Analysis of replication in Dutch Lifelines DEEP cohort

897 To analyze the overlap between SGVs detected in the Israeli cohort to those detected in the
898 Lifelines DEEP cohort, we ran ICRA and SGV-Finder independently on 1020 out of 1135
899 samples from the Lifelines DEEP cohort (EGA: EGAS00001001704) that had more than 10M
900 reads, and computed the percent of overlap between regions in both cohorts. To analyze
901 replication of associations between cohorts, we calculated for each SGV region in the Israeli
902 cohort, its presence / absence (deletion SGV) or abundance (variable SGV) in the Lifelines
903 DEEP cohort. We then tested the association of these regions with mean arterial pressure,
904 waist-to-hip ratio (stand in for the Israeli cohort waist circumference), body weight, BMI, fasting
905 glucose (stand in for the Israeli cohort median glucose), glycated hemoglobin, age, total and
906 HDL cholesterol measured in the Lifelines DEEP cohort, using a Mann-Whitney U test (deletion
907 SGVs) or the Spearman correlation (variable SGV).

908

909

910 Calculation of SGV conservation in cohabiting and related individuals

911 We calculated Spearman correlations between the deletion- and variable-SGV vectors of 39
912 pairs of individuals registered in our cohort as living in the same house. To calculate SGV
913 retention in first degree relatives, we calculated these correlations in 38 pairs of individuals
914 whose genomic SNP-based similarity⁴² was between 40 and 60%.

915

916 Functional enrichment analysis

917 This analysis was performed similarly yet separately to variable-SGVs, deletion-SGVs,
918 conserved regions, and regions significantly associated with the PTR of their harboring microbe.
919 For brevity, we collectively term them “regions”. We examined all gene annotations for all
920 microbial genomes analyzed using Ensembl functional annotation⁴³ available through
921 progenomes³⁶, and annotated orphan ORFs by mapping the protein sequence to all KEGG²⁶
922 protein sequences using DIAMOND⁶⁵ and selecting the top result with e-value<10⁻⁶ and at least
923 50% identity. We then used KEGG annotations to assign genes to modules, and calculated the
924 following textual categories by searching the progenomes gene function annotation using the
925 following regular expressions:

926 **Transposon:** transpos\|S*\|insertion\|Tra[A-Z]\|Tra[0-9]\|IS[0-9]\|conjugate transposon

927 **Plasmid:** relax\|S*\|conjug\|S*\|mob\|S*\|plasmid\|type IV\|chromosome partitioning\|chromosome segregation

928 **Phage:** capsid\|phage\|tail\|head\|tape measure\|antiterminatio

929 **Other HGT mechanisms:**

930 integrase\|excision\|S*\|exonuclease\|recomb\|toxin\|restrict\|S*\|resolv\|S*\|topoisomerase\|reverse transcrip

931 **Carbohydrate active:** glycosyltransferase\|glycoside

932 hydrolase\|xylan\|monooxygenase\|rhamnos\|S*\|cellulose\|sialidase\|S*\|ose\|S*\|s\|-

933)\|acetylglucosaminidase\|cellobiose\|galact\|S*\|fructose\|aldose\|starch\|mannose\|mannan\|S*\|glucan\|lyase\|glycosyltransfe

934 rase\|glycosidase\|pectin\|SusD\|SusC\|fructokinase\|galacto\|S*\|arabino\|S*

935 **Antibiotic resistance:** azole resistance\|antibiotic resistance\|TetR\|tetracycline resistance\|VanZ\|betalactam\|S*\|beta-

936 lactam\|antimicrob\|S*\|antibio\|S*

937 We searched for genes containing Pfam⁴⁴ modules with the keywords 'phage', 'prophage',
938 'transposon', 'conjugative transposon' using hmmscan (HMMER v3.1⁶⁶) with cutoff 1e-5. We
939 next counted, for each KEGG module, KEGG brite functional category, progenomes textual
940 gene category and Pfam keyword category the number of genes included and excluded in all
941 regions combined across all microbes. As the location of genes along microbial genomes is not
942 random p-values were calculated by permutations. In each permutation the sizes of both the
943 regions and the gaps between them were preserved but their ordering was randomly shuffled,
944 followed by examinations of genes in these regions and comparison of the number of included
945 and excluded gene in each KEGG module, brite functional category, etc., to the number found
946 without randomization. This was performed 1000 times.

947

948 Calculation of microbial growth rates

949 Microbial growth rates were quantified as peak-to-trough ratio (PTR) using the method and
950 software provided in ref.⁴⁵. PTRs were calculated for all the strains that were found to contain at
951 least one deletion-SGV and that whose reference genome sequence was complete (i.e., not
952 fragmented to contigs, as required by the PTR method⁴⁵), skipping the step of selecting a
953 representative strain per species. Mann-Whitney *U*-test was ran between PTRs of a bacteria in
954 samples in which it contained a certain deletion-SGV and PTRs of the same bacteria in samples
955 in which the same region was deleted, provided that at least 25 samples of each kind were
956 present.

957

958 SGV explorer

959 SGV explorer, presented in Figure S3 and accessible through
960 <https://genie.weizmann.ac.il/SGV/>, was created using bokeh for Python
961 (<http://bokeh.pydata.org>)

962

963 Code availability

964 ICRA, SGV-Finder, and the SGV Browser are available through github at
965 <https://github.com/segalab/SGVFinder>.

966

967 Data availability

968 The 7 strains samples used in Fig. 1C are available through ENA, accession ENA: PRJEB2519.

969 The 887 samples are publicly available through ENA, accession numbers ENA: PRJEB11532,

970 ENA: PRJEB17643.

971 **Figure Legends**

972

973 **Figure 1. Superior assignment of metagenomic reads using the Iterative Coverage-based**

974 **Read-Assignment (ICRA) algorithm.** (A) Illustration of our computational pipeline. (B) Bar-

975 plots (bar, mean; whiskers, standard deviation) of the ratio of correct read assignment per

976 taxonomy level with no assignment correction (blue) or following assignment correction with

977 ICRA (yellow), Kraken³⁸ (red) or MetaPhyler³⁹ (green). * two-sided Mann-Whitney U $p < 0.05$,

978 ** $p < 0.01$ (C) Dot-plot of the calculated relative abundance of 7 bacterial species in 100

979 samples, using either ICRA (yellow), MetaPhlAn2⁴⁰ (blue), or Bracken⁴¹ (red), as compared to

980 the true relative abundances. Inset shows a violin plot (white dot, median; black box, IQR) of

981 Bray-Curtis dissimilarities between the estimates of each method and the true abundances. **

982 two-sided Wilcoxon signed-rank $p < 0.01$ **** $p < 10^{-4}$

983

984 **Figure 2. Sub-Genomic Variation (SGV) is prevalent in the human microbiome, replicable**

985 **across cohorts and associated with specific functions.** (A) Heatmap showing the number of

986 subjects with SGVs (yellow color scale), the number of SGV regions (green color scale), the

987 mean SGV size (blue color scale) and the fraction of the genome that is variable (red color

988 scale), for each microbe analyzed, along with their phylogenetic tree. (B-C) Heatmap (B) and

989 swarm plot (C) showing the genomic length percentage of variable and deletions SGVs

990 replicated in the Lifelines DEEP cohort for each microbe analyzed. (D-E) Boxplot (box, IQR;

991 whiskers, 1.5*IQR) of the distribution of the correlations between variable- (D) or deletion-SGV

992 (E) across different subjects (green), within the same subject (blue), among cohabiting subjects

993 (yellow) and among pairs of siblings or parents/children (red). **- two-sided Mann Whitney U

994 $p < 0.01$ *** $p < 0.001$ **** $p < 10^{-5}$. (F-H) Fold change (x-axis) and statistical significance (Methods)

995 of the enrichment of functional KEGG modules in variable-SGVs (F), deletion-SGVs (G) and

996 conserved regions (Methods; H). (I) Difference in median value (x-axis) and statistical

997 significance in a Mann-Whitney U test (y-axis) comparing calculated bacterial growth rates
998 (PTR⁴⁵) under deletion versus retention of SGV.

999

1000 **Figure 3. SGVs are associated with disease risk and these associations replicate across**

1001 **cohorts.** (A-B) Heatmap of statistically significant correlations (Spearman $p < 0.001$, FDR

1002 adjusted at 0.1) between disease risk factors and variable-(A) or deletion-SGVs (B). Stars

1003 signify associations replicated (yellow), replicated using a different variable (orange) or

1004 reversed (gray) in the Lifelines DEEP cohort. Striped stars denote associations from the same

1005 bacteria that were collapsed for display purposes (see Figure S6 for full heatmap). (C) Boxplot

1006 (Box, IQR; whiskers, $IQR * 1.5$) of glycated hemoglobin (HbA1C%) in individuals harboring an 11-

1007 kbp deletion in the *E. rectale* genome (blue) and individuals with no deletion (maroon); p - Two-

1008 sided Mann-Whitney U test. (D) (top) Deletion rate across the cohort (y-axis) along a genomic

1009 region of *E. rectale* (x-axis). (bottom) gene locations (arrows) colored according to function

1010 (legend). (E) Scatterplot showing the correlation between the abundance of a 6-kbp variable-

1011 SGV in *R. torques* and weekly median glucose levels; p - Spearman correlation p -value. (F)

1012 (top) depiction of standardized variability (y-axis; plotted lines, percentiles 1, 25, 50, 75 and 99)

1013 along a genomic region of *R. torques* (x-axis). (bottom) gene locations (arrows) colored

1014 according to function (legend).

1015

1016 **Figure 4. A 31kbp deletion-SGV in *Anaerostipes hadrus* is associated with reduced**

1017 **weight.**

1018 (A) Boxplot (Box, IQR; whiskers, $IQR * 1.5$) of body weight in individuals harboring a 31-kbp

1019 deletion in the *A. hadrus* genome (blue) and individuals with no deletion (maroon). p - Two-

1020 sided Mann-Whitney U test. (B) Same as Fig. 3D for this genomic region of *A. hadrus*. (C)

1021 Depiction of the metabolic pathways encoded in the region, which turns inositol to the short-

1022 chain fatty acid butyrate. Note correspondence of enzyme commission (EC) numbers with panel
1023 B.

1024
1025 **Figure S1. ICRA reduces ambiguous assignments and noise.** (A) Boxplot (Box, IQR;
1026 whiskers, 10th and 90th percentiles) of ambiguous read assignment ratios of 887 samples^{20,30}
1027 mapped to a reference database of 3953 representative microbial genomes (Methods) before
1028 (blue) and after (yellow) ICRA correction. (B,C) Bar-plots (bar, mean; whiskers, standard
1029 deviation) of the ratio of incorrect read assignment per taxonomy level with no correction (blue)
1030 or following assignment correction with ICRA (yellow), Kraken (red) or MetaPhyler (green) for
1031 CAMI medium complexity (B; n=3) and low complexity (C; n=1) datasets. Note that MetaPhyler
1032 did not provide sub-species level read assignments.

1033
1034 **Figure S2.** (A-G) Dot-plot of the calculated relative abundance (y-axis) of *A. muciniphila* (A), *A.*
1035 *finnegoldii* (B), *B. faecium* (C), *C. flavigena* (D), *E. faecalis* (E), *L. gasseri* (F) and *S. cristatus* (G)
1036 in 100 samples, using either ICRA (yellow), MetaPhlAn (blue), or Bracken (red), as compared to
1037 the true relative abundances (x-axis). R^2 was calculated using Pearson correlation.

1038
1039 **Figure S3.** (A-B) Illustration of the online SGV explorer available at
1040 <http://genie.weizmann.ac.il/SGV/>, spanning the entire *R. torques* genome (A) and spanning a
1041 26-kbp region of the genome (B).

1042
1043 **Figure S4.** Fold difference (x-axis) and statistical significance (Methods) of the enrichment of
1044 functional KEGG modules in SGVs present in regions significantly associated with microbial
1045 growth dynamics.

1046

1047 **Figure S5. SGVs are associated with microbial growth rates.** (A) Boxplot (Box, IQR;
1048 whiskers, IQR*1.5) of microbial growth rates calculated using PTR⁴⁵ in individuals harboring a 7-
1049 segment deletion in the *E. eligens* genome (blue) and individuals with no deletion (maroon); (B)
1050 Genomic map of *E. eligens* with the 7 segments marked in yellow. (C) As in A for a 9-segment
1051 deletion-SGV in the *E. eligens* genome; (D) As in B with the 9 segments marked in orange.

1052

1053 **Figure S6.** Full heatmap of statistically significant correlations (Spearman $p < 0.001$, FDR
1054 adjusted at 0.1) between disease risk factors and variable-SGVs, depicting associations
1055 replicated (yellow star), replicated using a different variable (orange star) or reversed (gray star)
1056 in the Lifelines DEEP cohort.

1057

1058 **Figure S7.** (A) Boxplot (Box, IQR; whiskers, IQR*1.5) of BMI in individuals harboring a 4-kbp
1059 deletion in the *A. hadrus* genome (blue) and individuals with no deletion (maroon). (B) Same as
1060 Fig. 3D for this 4-kbp genomic region of *A. hadrus*. (C) Depiction of the genes encoded in the
1061 region, which encode key enzymes in the folate biosynthesis pathway. Note correspondence of
1062 enzyme commission (EC) numbers with panel B. (D) Boxplot (Box, IQR; whiskers, IQR*1.5) of
1063 total cholesterol in individuals harboring an 18-kbp deletion in the *R. intestinalis* genome (blue)
1064 and individuals with no deletion (maroon). (E) same as Fig. 3D for a 10-kbp stretch of the 18-
1065 kbp region in *R. intestinalis*. (F) Boxplot (Box, IQR; whiskers, IQR*1.5) of BMI in individuals
1066 harboring an 8-kbp deletion in the *C. comes* genome (blue) and individuals with no deletion
1067 (maroon). (G) Same as Fig. 3D for this 8-kbp genomic region of *C. comes*. p - Two-sided Mann-
1068 Whitney U test.

1069

1070 **Figure S8.** Replication of deletion and variable regions depicted in Fig. 3, 4 and S7 between the
1071 Israeli (yellow) and Dutch Lifelines DEEP (blue) cohorts.

1072

1073 **Figure S9.** (A-C) Boxplot (Box, IQR; whiskers, IQR*1.5) of waist circumference (A), BMI (B) and
1074 HDL cholesterol (C) in individuals of the Israeli cohort harboring the 31-kbp deletion in the *A.*
1075 *hadrus* genome depicted in Fig. 4 (blue) and individuals with no deletion (maroon). (D) Boxplot
1076 (Box, IQR; whiskers, IQR*1.5) of BMI in individuals of the Dutch Lifelines DEEP cohort
1077 harboring the same 31-kbp deletion in the *A. hadrus* genome (blue) and individuals with no
1078 deletion (maroon). p - Two-sided Mann-Whitney U test.