Social relationships, social isolation, and the human gut microbiota

Short title: Social relationships and the gut microbiota

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ABSTRACT

Social relationships shape human health and mortality via behavioral, psychosocial, and physiological mechanisms, including inflammatory and immune responses. Though not tested in human studies, recent primate studies indicate that the gut microbiome may also be a biological mechanism linking relationships to health. Integrating microbiota data into the 60-year-old Wisconsin Longitudinal Study, we found that socialness with family and friends is associated with differences in the human fecal microbiota. Analysis of spouse (N = 94) and sibling pairs (N = 83) further revealed that spouses have more similar microbiota and more bacterial taxa in common than siblings, with no observed differences between sibling and unrelated pairs. These differences held even after accounting for dietary factors. The differences between unrelated individuals and married couples was driven entirely by couples who reported close relationships; there were no differences in similarity between couples reporting somewhat close relationships and unrelated individuals. Moreover, the microbiota of married individuals, compared to those living alone, has greater diversity and richness, with the greatest diversity among couples reporting close relationships, which is notable given decades of research documenting the health benefits of marriage. These results suggest that human interactions, especially sustained, close marital relationships, influence the gut microbiota.

INTRODUCTION

Social relationships exert a sustained influence on human health and mortality with social isolation having strong negative consequences and high levels of social integration far exceeding the protective effects on mortality of individual level behaviors such as smoking cessation or maintaining a normal weight [1,2]. Research in the social sciences has shown that individuals who cohabitate in marriage and marital like relationships have better health than do unpartnered adults[3]. For both social relationships generally, and marriage specifically, health benefits are largely achieved in the context of high quality relationships. The robust links between these relationships and health are related to stress, behaviors, and psychosocial resources, among other factors [2]. In part, social support may impact one's health by reinforcing healthy habits, reducing the impacts of stress, and preventing the use of unhealthy "self-medications" like smoking and drinking [2]. Additional research points to stress-related biological processes that may also contribute to the positive impacts of social relationships through changes in inflammatory processes, metabolic syndrome, and neurological functioning [4,5].

Recent work in the field of microbiology points to another possible biological mechanism linking human relationships and health: the microbiome. The microbial communities that inhabit mammals have profound effects on biology and health [6]. Gastrointestinal (GI) microbial communities impact host health by modulating the epigenome [7], brain function [8], and metabolism of drugs and nutrients [9] as well as impacting immune system function [10] and development [11]. While the microbiota reaches an adult-like configuration by three to five years of age [12], considerable variation exists between adults [13], and differences are mediated by a number of factors. Most notable among these are diet [14] and host genetics [15], which also correlate with health. An individual's microbiota structure (*i.e.* relative abundance) and composition (*i.e.* who's there) can

70 change rapidly in response to inputs like diet [16] and antibiotics [17]. Nonetheless, there is evidence
71 that an individual's microbiota remains relatively stable over many years [18–20], perhaps in part
72 because a person's behaviors also tend to be consistent over many years.

73 While a number of factors like diet are known to impact both the microbiota and health [21],
74 less is known regarding social relationships. Most existing research has focused on animal models,
75 which has produced compelling evidence that social interactions, via a range of different types of
76 physical contact, influences the gut microbiota through microbial sharing between individuals [22–
77 26]. Additionally, states of isolation, such as maternal neglect, influence the gut microbial
78 composition in animal models [27] at least in part through stress [28,29]. Thus, the gut microbiota may
79 play a role in some of the long-term health effects of social relationships.

80 But despite this tantalizing evidence, studies in human populations remain relatively small
81 in number [30]. There are a few studies exploring how mother-infant interactions influence the
82 development of the infant's gut microbiome and even how broader social interactions influence
83 the milk microbiome [31,32]. In terms of adults, there is evidence regarding the influence of
84 cohabitation, may influence the gut microbiome. A few recent studies have found that individuals
85 living together had more similar gut [33] and skin [33,34] microbiota. Interestingly, however, another
86 study found that married cohabitating couples had no more similarity in the composition of their
87 gut microbiota than did unrelated individuals [35].

88 Thus, while it does appear that living together may influence the gut microbiome, human
89 studies have not investigated how adult relationships, rather than just simply living in the same
90 space, may influence the gut microbiome. The quality of the relationship may matter. Closer
91 relationships likely lead to even closer shared environments, via mechanisms such as time spent
92 physically together. Indeed, one recent study of wild baboons found that close partners within

social groups had more similar gut microbiotas [36]. Studies have also have not more generally compared how living alone versus living with an intimate partner influences the gut microbiome; individuals living alone are on average, de facto, more socially isolated than those living with someone, and animal studies have generally shown that social isolation leads to decreased microbial diversity [22,37–39]. Though causality is not certain, decreased microbial diversity is associated with obesity, cardiac disease, and type 2 diabetes, and a range of other inflammatory disorders [40–47]. More broadly, there is extensive evidence that cohabitating couples in later life have substantially improved physical and psychological well-being compared to single adults [48–50]. Thus, similar mechanisms might explain some of the variance in findings in humans.

An important hindrance to research examining social relationships and the GI microbiota is the availability of human samples with sufficiently well-characterized life course measures of broader social environments and conditions. Thus, most microbiological research in this field is based on animal models [22–25]. However, there are now a wide array of well-characterized longitudinal studies in the social sciences that have generated decades of research documenting relationships between broader social environments and mortality [5,51–54]. These data can provide a platform for studies of the human microbiota to advance knowledge for both social scientists and microbiologists, including whether social conditions influence the gut microbiota and whether the gut microbiota is a mediating biological mechanism explaining how social conditions influence health.

Here, we leverage a multidisciplinary collaboration to investigate the links between human interaction, the microbiota, and human health. We utilized data in the nearly 60-year Wisconsin Longitudinal Study (WLS) [54], which constitutes a random sample of 1 in 3 1957 Wisconsin high school graduates (N = 10,317), as well as selected spouses and siblings surveyed periodically

116 during their adult life. We correlate the fecal microbiota of 408 older individuals (58 – 91 yo) from
117 WLS with extensive health and behavioral data, as well as compare spouse and sibling pairs within
118 the cohort. Overall, this project demonstrates the promise of joint participation between social
119 scientists and microbiologists in efforts to more fully understand the gut microbiota and its impacts
120 on human health.
121
122 **RESULTS and DISCUSSION**
123 We employed 16S rRNA gene sequencing to characterize the fecal microbiota of 408 individuals,
124 including Wisconsin Longitudinal Study (WLS) graduates (N = 179, 76 ± 0.5 years old), siblings
125 of graduates (134, 74 ± 6.4), spouses of graduates (63, 76 ± 3.7), and spouses of siblings (32, 73 ±
126 6.1). We then correlated these communities to longitudinal survey data collected from 1957 to
127 2015 as part of WLS [54]. A total of 24.5 million high-quality sequences were obtained for 408 fecal
128 samples (60,000 ± 19,000 SD sequences per sample) after quality filtering in mothur. All samples
129 achieved sufficient coverage as determined by Good's coverage > 99% (Dataset S1). For more
130 details on this data collection see [55]. We note that all analyses have adjusted potential confounders
131 including age, sex, antibiotics, diet (dietary protein), and chronic conditions (diabetes and heart
132 disease) unless stated otherwise. Dietary data reflects the three days prior to the fecal sample
133 collection. We tested a wide array of specific dietary variables, but consumption of dietary protein
134 was the only one robustly associated with microbial composition.
135
136 **Social interactions and the human fecal microbiota** In the WLS graduate cohort, we have
137 identified factors correlated with gastrointestinal (GI) microbiota. These factors include sex,

138 antibiotics, dietary proteins, high blood sugar and heart disease (Fig. 1, Fig. S1). These factors
139 were reported in the previous literature [56,57].
140
141 Human interactions were associated with differences in gut microbiota and diversity. Specifically,
142 we found that individuals that were cohabitating with a spouse or partner had more similar
143 microbiota composition with their cohabitating spouse/partner as well as higher diversity and
144 richness than unmarried, non-cohabitating individuals (unweighted UniFrac $P = 0.029$ Shannon P
145 $= 0.005$, Chao $P = 0.011$, Fig. 2). Since all cohabitating pairs were male-female and sex was a
146 strong determinant of the microbiota in this study ($P < 0.001$, Table S1), increased diversity may
147 be partially due to sustained exchange of microorganisms between the sexes, though we were not
148 able to test this given that because there were no same sex couples in these data. Increases in
149 diversity seen here are consistent with a previous cohabitation study in pigs [58] and may have
150 implications for human health, as previous work indicates that increased gut microbial diversity is
151 associated with lower risks of irritable bowel syndrome (IBS), Crohn's disease, ulcerative colitis,
152 and other gastrointestinal (GI) related afflictions [59].
153 Social interactions with relatives and friends were stronger predictors of gut microbial
154 diversity in non-cohabiting individuals than cohabiting spouses/partners (unweighted UniFrac $P =$
155 0.0030, Shannon $P = 0.042$, Chao $P = 0.063$, Fig. S2) (Table S1). Here, social interactions were
156 defined as the sum of "How many times during the past four weeks have you gotten together with
157 relatives/friends?" The associations may have been weaker for cohabitating spouses due to their
158 higher microbial diversity; ecological theory supports that diverse communities are more resilient
159 and resistant to invasion by new species [60]. Thus, one explanation for these differential associations
160 is that the more diverse microbiotas of individuals already cohabitating with a spouse may not

161 have been as strongly influenced by increasing social interactions while the less diverse
162 microbiotas of those living alone were more strongly influenced by invasion of new species
163 through social exposures. It's also possible that cohabitating couples share the same friends and
164 socialize together with these friends. However, factors contributing to the resilience of the human
165 gut microbiota require further exploration to confirm this hypothesis.
166
167 **Spouses have more similar microbes than siblings and unrelated individuals** Previous studies
168 have established that the GI microbiota reaches an adult-like configuration by 3 to 5 years of age
169 [18,61,62] and that during adulthood, communities are stable on the time scale of years [19,20]. Thus,
170 microbial communities established in early life may persist and, aside from extreme perturbation,
171 remain stable across one's adult lifetime. Our analyses, comparing sibling pairs versus couple pairs
172 versus unrelated pairs, challenge the assumption that microbial communities established in early
173 life will be largely unperturbed in later life (for details, see METHODS, Statistical Analysis for
174 spouse and siblings). In fact, we find no evidence for a remaining influence of early life on the
175 composition of the gut microbiota among older adults. In this older cohort, spouses were more
176 similar than unrelated subjects (unweighted UniFrac P = 3.2E-5) or sibling pairs (unweighted
177 UniFrac P = 0.033, Fig. 3). Further, the length of the cohabitating marital relationship was
178 positively correlated with similarity (unweighted UniFrac, P = 0.031) In contrast, siblings were
179 no more similar than unrelated pairs by any beta-diversity metric (P > 0.3, Fig. 3A, Fig. S3A, D,
180 G) (Table S1). Thus, adult factors like marriage with cohabitation (spouses) appear to have a
181 greater influence on the adult gut microbiota than early-life environment or genetics (siblings).
182 We also found no evidence that the physical proximity of siblings—as measured both by physical
183 distance between siblings—influenced gut microbial similarity.

184 This is further supported by our findings that childhood farm status was not associated with
185 microbial richness (Chao $P = 0.342$) while working on a farm as an adult correlated with higher
186 richness (Chao $P = 0.005$). Farm-driven differences in the microbiota are of particular interest,
187 because adolescents that grew up on a farm have more diverse microbial communities [63] and
188 reduced risk of asthma and other atopic diseases both during childhood [64] and as adults [65]. Given
189 the results here, it appears that the microbially-driven protective effects of early farm exposures
190 are not due to the persistence of protective microorganisms acquired in early-life. Protection may,
191 instead, be conferred by immune development and training by early-life microbes as suggested
192 previously [66].

193 Our results are also in contrast with previous work showing that genetically related
194 individuals harbor more similar microbial communities than unrelated individuals, regardless of
195 current cohabitation [35,67–69]. However, these previous studies investigated children [67], young adults
196 [35,68], or a wide age range [69], and therefore, cumulative changes across a lifetime may not have
197 reached a level sufficient to overcome early-life factors impacting the microbiota. Additionally,
198 sibling pairs in other studies were twins [35,67–69], and many focused on monozygotic twins (same
199 sex and age) [35,68,69] as opposed to this study where siblings were often of opposite sexes (43%) and
200 ranged from less than a year to 18 years apart in age. Also, the unrelated group in this study may
201 have exhibited higher homogeneity than unrelated groups in other studies, because most grew up
202 in and/or currently live in the state of Wisconsin. Thus, compared to previous studies, siblings
203 were likely less similar and unrelated pairs more similar across our cohort. Furthermore, genetic
204 effects on the microbiota are often small [69] and detection may require a larger human cohort than
205 used here. Taken together, these factors may have contributed to the lack of significant differences

206 observed between sibling and unrelated groups even though average sibling beta-diversity was
207 intermediate between spouses and unrelated individuals.
208
209 **Increased microbial similarity, diversity, and richness in closer relationships** For both spouse
210 and sibling relationships, microbiota similarity was associated with self-reported relationship
211 closeness (unweighted UniFrac $P = 0.0079$). Closeness was measured by participant responses to
212 "How close are you and your current spouse/sibling?" on a scale of not at all (1) to very (4). Due
213 to the small sample sizes in the categories "Not very" (N=13) and "Not at all" (N=4), we combined
214 these two groups and named the new group "Not" close. Across spouses and siblings, individuals
215 in very close relationships harbored more similar gut microbial communities to these close social
216 partners than those in not very close relationships (Fig. 3B), though the relationships were not
217 significant within spousal and sibling pair groups (Fig. 3C). However, the differences between
218 spouses and unrelated individuals, in terms of closeness (Fig. 2), was driven by spouses reporting
219 very close relationships. The similarity of gut microbial communities did not differ between
220 unrelated individuals and couples reporting somewhat close relationships (Table S2). Moreover,
221 the enhanced diversity and richness in cohabitating couples versus individuals living alone (Fig.
222 1), was driven by couples who reporting having very close relationships. There was no difference
223 in diversity or richness between couples reporting only somewhat close relationships and
224 individuals living alone (Table S3).
225 While diet is often correlated with the GI microbiota [70], closeness points to the less well-
226 understood contributions of human interactions and shared behaviors. Close proximity and
227 frequent physical contact were correlated with microbiota similarity among primates with direct
228 microbial sharing between individuals contributing to similarity [22,23]. In this study, relationship

229 closeness may represent a summative measure of time spend together, physical affection, and other
230 human interactions with the potential to result in microbial sharing. Indeed, there is evidence that
231 the salivary microbiome influences the gut microbiome and the salivary microbiome may be
232 influenced by kissing [71,72]. In these data, this is supported by the fact that spouses had more
233 operational taxonomic units (OTUs, a proxy for microbial species) in common (30.4 ± 7.32%)
234 than siblings (26.4 ± 7.47%, t-test P = 4.39E-04) (Dataset S2). Also, when comparing the spouse
235 and sibling pair within a family represented in this dataset, a person tended to have more OTUs in
236 common with his or her very close spouse (25.4 ± 7.9%) than his or her very close sibling (22.2 ±
237 6.4%, N = 12 families, P = 0.074, Fig. 3D). This is also true when comparing very close spouses
238 (22.9 ± 5.8%) and somewhat close siblings within a family (20.6 ± 5.5%, N = 17 families, P =
239 0.027, Fig. 3E).
240
241 **Shared taxa with close human relationships**. In general, highly abundant genera and OTUs were
242 shared between many spouse and sibling pairs while less abundant shared taxa were specific to
243 one pair type and shared by a small number of pairs within that type (Dataset S3). OTUs that were
244 commonly found among spouses or siblings (> 50% of pairs) but rare in the unrelated dataset (<
245 70% individuals, < 49% unrelated pairs) may represent bacterial species easily shared by close
246 human interaction. These OTUs were predominately from the phylum Firmicutes (16 of 22 OTUs)
247 with representatives of families Lachnospiraceae and Ruminococcaceae (Dataset S4).
248 Interestingly, most of these potentially shared OTUs were from strictly anaerobic taxa, indicating
249 that persisting in an oxygen-rich environment in-between hosts may not be a limiting factor in very
250 close human relationships. Transmission, in these cases, could be mediated by direct contact
251 similar to mechanisms of vertical transmission from mother to child [73].

252 Taxa commonly associated with reduced disease incidence or severity like *Akkermansia*
253 *muciniphila* [74], *Bifidobacterium* spp. [75,76], *Collinsella aerofaciens* [76], and *Ruminococcus bromii* [77]
254 as well as potentially harmful taxa like *Clostridium spiroforme* [78,79] were often present in both
255 persons in a spouse or sibling pair. Several of these potentially shared OTUs were associated with
256 disease incidence in the larger dataset. In particular, *Ruminococcus bromii*, *Lachnospira* spp. and
257 unclassified Ruminococcaceae and Lachnospiraceae OTUs were less abundant in those with high
258 blood sugar (Fig. 4, Dataset S4). These results are in contrast to previous reports of more abundant
259 Ruminococcaceae/*Ruminococcus* [80,81] and Lachnospiraceae [80] associated with diabetes in humans
260 and may point to important differences in the impacts of the microbiota on metabolic health in
261 older populations. Overall, though, this indicates that GI microbial species with the potential to
262 impact host health may be shared by close human interactions. However, it cannot be discounted
263 that these apparent health associations may be mediated by diet as those with high blood sugar
264 often consume specific diets to manage disease.

265 Overall, our findings indicate that in order to understand environmental influences on the
266 gut microbioata, we must now consider the many microbiotas with which this individual interacts.
267 Socialness with family and friends is associated with differences in the fecal microbiota. These
268 differences held even after accounting for dietary factors. Thus, it is possible that relationships
269 with others may influence the gut microbiota and consequent health outcomes, either through
270 direct microbial transfer or reinforcement of healthy microbiota behaviors. Indeed, we found not
271 only that married couples had more similar gut microbiota but also that the microbiota of married
272 individuals, compared to those living alone, has greater diversity and richness. Key to both of these
273 findings, however, was that they were driven by individuals reporting that they were very close to
274 their spouse as opposed to somewhat close. Close marriage relationships had a stronger influence

275 than the shared genetic factors and early life environments among siblings. This finding is
276 interesting, in part, because it parallels an extensive body of evidence demonstrating robust links
277 between high quality marriages and morbidity and mortality. Future work could attempt to
278 disentangle the mechanisms linking close relationships to microbial composition. For example,
279 while we did not find evidence that shared diet was primarily responsible for these findings, we
280 could not test precise frequencies of physical contact and intimacy as an alternative explanatory
281 mechanism. Importantly, the types of physical contact and intimacy change over the life course,
282 with sexual intimacy becoming far less frequent in later life, but other kinds of intimate physical
283 contact remaining important. Overall, these results provide support for the gut microbiome as a
284 possible mediating pathway between social relationships, especially marriage, and health and
285 mortality. These findings, in the context of the robust body of evidence linking social relationships
286 to human morbidity and mortality, provide fodder for further work examining the role of the gut
287 microbiome as a possible biological mediator in these relationships [32]. Further microbiota work
288 across time in a more diverse population should be undertaken with the many longitudinal social
289 science studies currently underway in an effort to increase our understanding of the complex
290 interactions between human behavior, the microbiota, and health.
291

292 **METHODS**

293 **Wisconsin Longitudinal Study (WLS)** WLS is based on a one-third sample of all 1957
294 Wisconsin high school graduates (N = 10,317) as well as selected siblings and spouses [54].
295 Graduates originally enrolled with an in-person questionnaire upon graduating high school in
296 1957, which was followed by data collection in 1964, 1975, 1992, 2004, and 2011. Siblings were
297 surveyed in 1977, 1994, 2005, and 2011; spouses were surveyed in 2004 or 2006. The content of

298 WLS surveys changed to reflect the participants' life course with an education focus in the initial
299 data collection, familial and career outcomes in young adulthood / midlife, and health, cognitive
300 functioning, psychological well-being, non-work activities, caregiving, bereavement, social
301 support, and end-of-life preparations in later rounds. WLS data collection was approved by the
302 Institutional Review Board (IRB) at the University of Wisconsin-Madison (2014-1066, 2015-
303 0955). Informed consent, the content and procedures of which were included in the IRB approval,
304 was obtained from participants. All methods were performed in accordance with relevant
305 guidelines and regulations.
306
307 **Study design** A total of 500 individuals were randomly drawn from the full WLS dataset
308 constrained based on the following: 1) participated in the 2011 interviews; 2) lived in one of 10
309 counties in Wisconsin that included both northern rural counties and southern more urban counties;
310 and 3) were part of a sibling pair. Individuals were removed from the study if they did not give
311 consent, their sample did not arrive for processing chilled, but not frozen, within 48 hrs of
312 collection, or their sample did not yield at least 10,000 sequences for analysis. This resulted in 408
313 individuals being included in this study.
314 An additional survey was administered at the time of fecal sampling, which detailed dietary
315 data from the prior three days, prescription/antibiotic use, current living situation, and additional
316 health information. This as well as selected data from the larger WLS study focused on health,
317 spouse/sibling relationships, and social interactions were used in this study (Text S1). Data,
318 documentation, and other materials are accessible at http://www.ssc.wisc.edu/wlsresearch/.
319 Access to the full dataset can be obtained through wls@ssc.wisc.edu.
320

321 **Sample collection** Stool samples were collected by participants in November 2014, January 2015,
322 or April 2015 following provided instructions (Text S2). Participants stored samples at ~4 °C in
323 their refrigerator or in a NanoCool box (Albuquerque, NM) with cooling cartridge and customized
324 foam insert, supplemented with a single ice pack. Interviewers picked-up samples from
325 participants within 24 hours of collection and shipped samples in fresh NanoCool boxes for arrival
326 at UW-Madison within 48 hours of collection. Upon arrival, an aliquot of feces was collected for
327 DNA extraction and immediately stored at -80°C until further processing. The use of WLS and
328 fecal microbiota data were approved by the Institutional Review Board at the University of
329 Wisconsin-Madison (2017-0600).
330
331 **DNA extraction** Genomic DNA was extracted from fecal aliquots using a bead-beating protocol
332 [45]. Briefly, feces (~100 mg) were re-suspended in a solution containing 500 µl of extraction buffer
333 [200 mM Tris (pH 8.0), 200 mM NaCL, 20 mM EDTA], 210 µl of 20% SDS, 500 µl
334 phenol:chloroform:isoamyl alcohol (pH 7.9, 25:24:1) and 500 µl of 0.1-mm diameter
335 zirconia/silica beads. Samples were mechanically disrupted using a bead beater (BioSpec Products,
336 Barlesville, OK; maximum setting for 3 min at room temperature), followed by centrifugation,
337 recovery of the aqueous phase, and precipitation with isopropanol. QIAquick 96-well PCR
338 Purification Kit (Qiagen, Germantown, MD) was used to remove contaminants. Isolated DNA was
339 eluted in 5 mM Tris/HCl (pH 8.5) and was stored at -80 °C until further use.
340
341 **Sequencing** PCR was performed using universal primers flanking the variable 4 (V4) region of
342 the bacterial 16S rRNA gene [82]. In one reaction per sample, 10 - 50 ng DNA, 10 µM each primer,
343 12.5 µl 2X HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA), and water to 25 µl

344 were used. Cycling conditions were initial denaturation of 95 °C for 3 min followed by 25 cycles
345 of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension of 72 °C for 5 min.
346 PCR products were purified with the QIAquick 96-well PCR Purification Kit (Qiagen,
347 Germantown, MD, USA). Samples were quantified by Qubit Fluorometer (Invitrogen, Carlsbad,
348 CA, USA) and equimolar pooled. The pool plus 5% PhiX control DNA was sequenced through
349 the U. of Wisconsin-Madison Biotechnology Center with the MiSeq 2x250 v2 kit (Illumina, San
350 Diego, CA, USA) using custom sequencing primers [82]. All DNA sequences are available upon
351 institutional review board (IRB) or other ethics board approval through wls@ssc.wisc.edu.
352
353 **Sequence clean-up** All sequences were demultiplexed on the Illumina MiSeq. Sequence clean-
354 up and processing was performed with mothur v.1.36.1 [83] following a protocol similar to [82].
355 Briefly, paired-end sequences were combined into contigs with default parameters (match bonus
356 = 1, mismatch penalty = -1, gap penalty = -2, gap extend penalty = -1, insert quality ≥ 20,
357 mismatch quality difference ≥ 6). Poor-quality sequences, including those with ambiguous base
358 pairs, homopolymers greater than 8, or outside 200 – 500 bp in length, were discarded.
359 Sequences were then aligned to the SILVA 16S rRNA gene reference alignment database [84] and
360 trimmed to the V4 region. To reduce sequencing error, sequences with 2 or fewer differences
361 were pre-clustered. Chimera detection and removal were performed using UCHIME [85]. Final
362 sequences were then classified to the GreenGenes database [86]. Singletons were removed to
363 facilitate downstream analyses. All sequences were grouped into 98% operational taxonomic
364 units (OTUs) by uncorrected pairwise distances and average neighbor clustering in mothur.
365 Clustering performed on uncorrected pairwise distances revealed no differences in clusters at 97
366 vs 98% similarity. Therefore, the stricter cutoff was reported Coverage was assessed by Good's

367 coverage, and then samples were normalized to whole number counts by percent
368 relative abundance to approximately 10,000 sequences per sample (9,914 - 10,061 after
369 rounding.
370
371
372 **Statistical analysis for graduates** Graduates were assessed separately from siblings and spouses
373 to avoid potential interactions, and the graduate subset was not significantly different from other
374 groups (PERMANOVA *P* Bray-Curtis $P = 0.56$, Jaccard $P = 0.57$, weighted UniFrac $P = 0.33$,
375 unweighted UniFrac $P = 0.24$). Alpha-diversity was assessed with Shannon's diversity and Chao's
376 richness calculated in mothur. Differences in alpha-metrics were assessed in R v3.3.2 [87] by linear
377 regression with the Benjamini-Hochberg correction for multiple comparisons across each metric.
378 Beta-diversity was assessed for Bray-Curtis, Jaccard, weighted, and unweighted UniFrac metrics
379 with results shown for unweighted UniFrac unless otherwise noted. Differences in beta-diversity
380 were tested with permutational analysis of variance (PERMANOVA, adonis) in the vegan package
381 [88] with the Benjamini-Hochberg correction for multiple comparisons across each metric and a
382 maximum of 5000 permutations. The factors that associate with the microbiome (*i.e.* age, sex,
383 antibiotics, diet, high blood sugar, and heart disease [56]) were adjusted for in regression models as
384 potential confounders. Beta-diversity was visualized by non-metric multidimensional scaling
385 (nMDS) plots with arrows from significant variables (PERMANOVA) fitted to the ordination
386 using maximum correlation (envfit, vegan). All tests were assessed at significance $P < 0.05$ and
387 trends $0.05 < P < 0.1$.
388
389 **Statistical analysis for spouses and siblings**

390 For the spouse and sibling similarity analysis, the unit of the observation is the pair (*i.e.* spouse,
391 sibling, or unrelated pair defined below) and the variables used in the analysis are distance in
392 individual measurements between the two members of the pair. Specifically, beta-diversity metrics
393 were used to quantify the distance in microbial composition and absolute difference were
394 calculated to quantify the distance in all the other variables (*e.g.* age, sex, diet). We sampled
395 unrelated pairs from the data in order to compare the spouse or sibling pair with unrelated pairs.
396 In particular, the unrelated individuals cannot be siblings, spouses, or in-laws, and each unrelated
397 pair will match the corresponding spouse or sibling pair in sex and antibiotics usage. Beta-diversity
398 distances were compared among spouse, sibling, and unrelated pairs using linear regression while
399 adjusting for the distance in age, sex, dietary protein, health conditions (if available). P-values
400 were averaged across 1000 rounds of unrelated pair sampling. For closeness analysis, we removed
401 age and sex from the model because the two variables are highly correlated with pair type (*i.e.*
402 sibling/spouse pair can be accurately classified using the difference of the age or sex between the
403 two members of the pair). For comparing OTU sharing among spouse and sibling within a family,
404 we used mixed-effect models to account for family clustering. All tests were assessed at
405 significance $P < 0.05$ and trends $0.05 < P < 0.1$. All code is available at
406 https://github.com/kdillmcfarland/WLS.
407

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604

605 **AUTHOR CONTRIBUTIONS**

606 FER and PH designed research. JHK, RLK, and TS completed sample processing and sequencing.
607 AP contributed to the conceptual and analytic approach to conducting the analyses. KADM, ZZT,
608 and GC analyzed the data. KADM, ZZT, GC, FER, and PH contributed to the final manuscript.
609 Alberto Palloni[5]

610

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621

622 The authors declare that we have no competing interests as defined by Nature Research, or other
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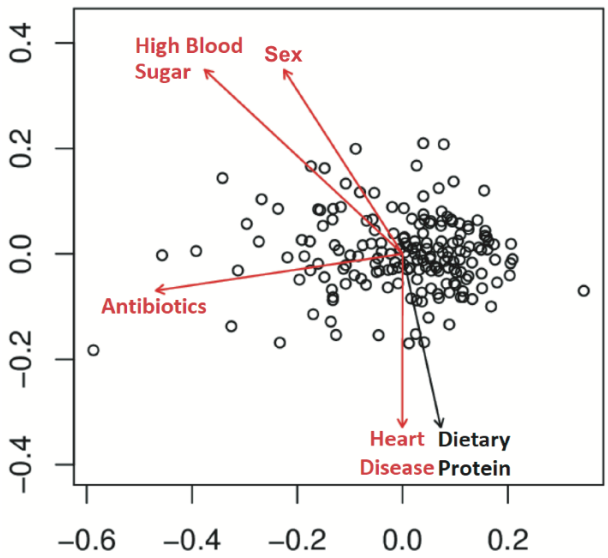
624 **FIGURES**



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626 **Figure 1.** Factors associated with the overall fecal microbiota. Non-metric multidimensional
627 scaling (nMDS) of unweighted UniFrac for all graduates (N = 179). Variables found to be
628 significant (PERMANOVA $P < 0.05$, red) and trends ($0.05 < P < 0.1$, black) are shown as fitted
629 arrows. Arrows point toward increasing values (dietary protein), toward affirmative responses
630 (high blood sugar, antibiotics, heart disease), or from male to female (sex).

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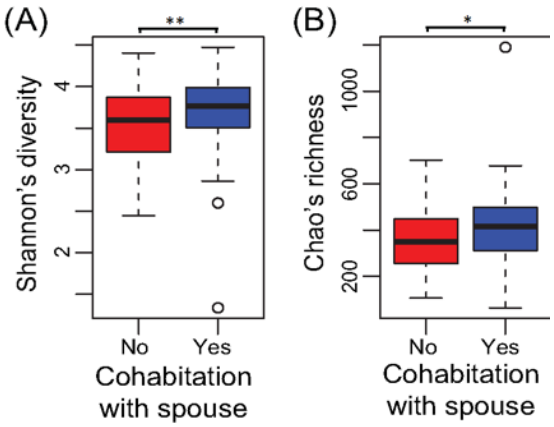

Figure 2. Cohabitation is associated with increased alpha-diversity. Boxplots of (A) Shannon's diversity and (B) Chao's richness of graduates that are (blue) or are not (red) cohabitating with a spouse or partner. All spouses/partners were cohabitating while all non-cohabitating individuals were unmarried. **P < 0.01, *P < 0.05

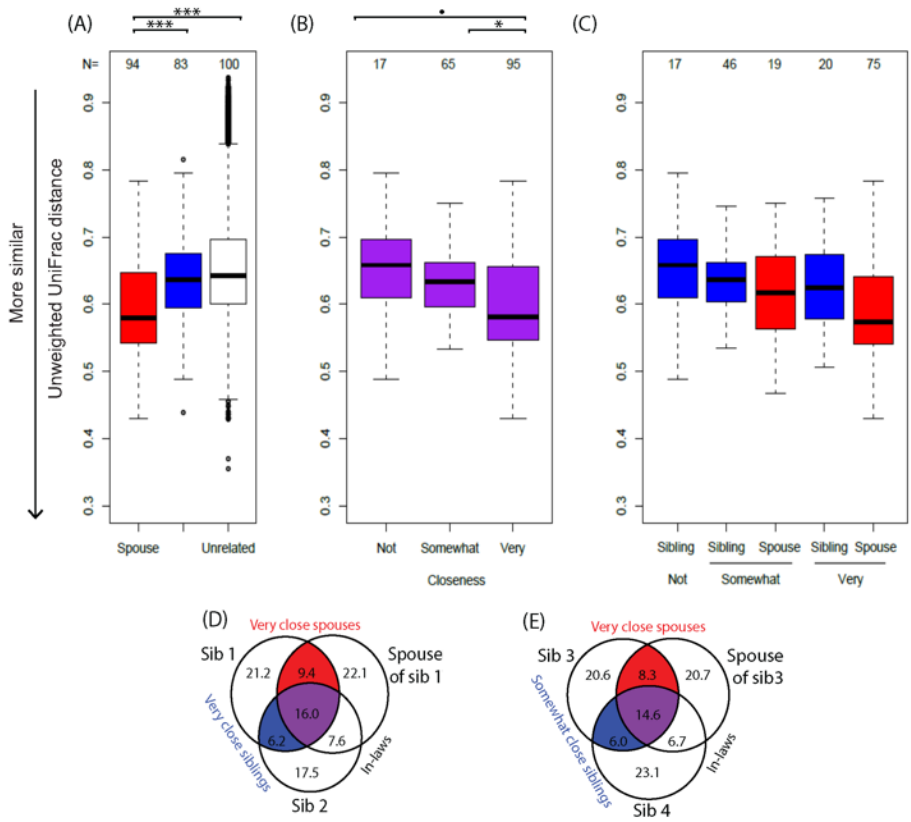

Figure 3. Microbial sharing in spouse and sibling relationships. Unweighted UniFrac distances of (A) spouse, sibling, and unrelated pairs, (B) spouses and siblings grouped by relationship closeness, and (C) spouses and siblings separated by relationship closeness. Groups (A) were compared in linear regression model adjusting for potential confounders (*e.g.* age, sex, diet, health conditions). P-values were averaged across 1000 rounds of unrelated pair sampling. Closeness groups (B,C) were compared in linear regression models adjusting for potential confounders. (D, E) Average percentages of shared OTUs within family groups including a related spouse and sibling pair. Families included those with both very close spouses and siblings (D, N = 12) and those with very close spouses and somewhat close siblings (E, N = 17). Percentages are of the total number of OTUs across all three individuals, and circle sizes are proportional to total percentages represented. ***P < 0.001, **P < 0.01, *P < 0.05, •P< 0.1

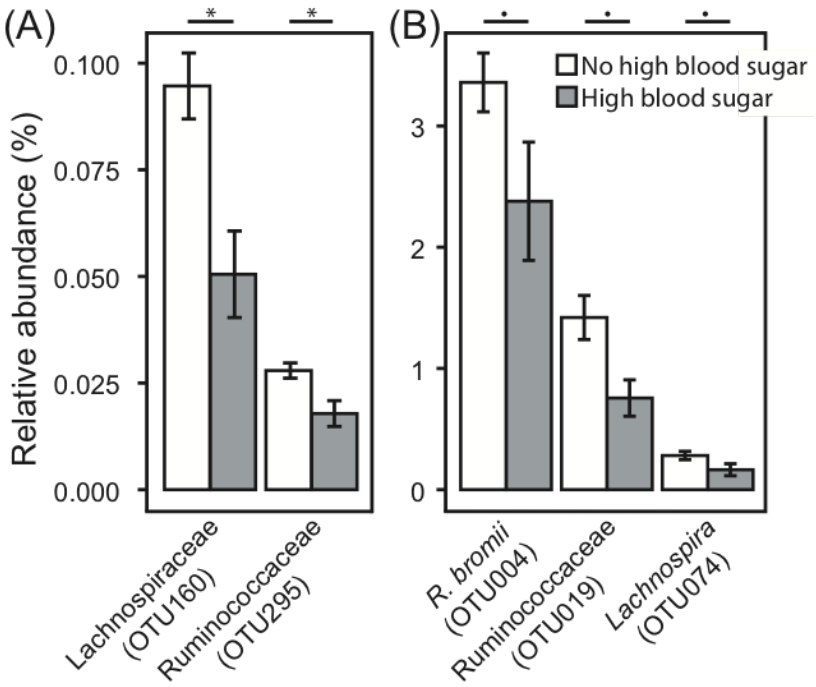

Figure 4. Percent relative abundance of OTUs that are commonly shared between spouses and that differed between those with (grey) and without (white) high blood sugar. (A) Low abundance and (B) more highly abundant OTUs. Means with standard error bars are shown. Kruskal-Wallis FDR *P < 0.05, •P < 0.1