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]. For more details on this data collection, see [55]. A total of 24.5 million
128 high-quality sequences were obtained for 408 fecal samples (60,000 ± 19,000 SD sequences per
129 sample) after quality filtering in mothur. All samples achieved sufficient coverage as determined
130 by Good's coverage > 99% (Dataset S1).
131 In the WLS graduate cohort, we identified several factors correlated with gastrointestinal
132 (GI) microbiota including sex, antibiotics, dietary protein, high blood sugar, and heart disease (Fig.
133 1, Fig. S1, Table S1). These factors were reported in the previous literature [57,58] with diet playing
134 a particularly strong role [14,16,56]. Thus, we assessed diet across a number of measures including
135 habitual intake of protein, vegetables, and fruits (Text S1) during the year prior to the fecal sample
136 collection (for details, see METHODS, Statistical analysis for graduates). While overall dietary
137 dissimilarity (Bray-Curtis and Jaccard) across these three categories correlated with gut microbiota
138 dissimilarity, only the total frequency of dietary protein consumption was robustly associated with

139 microbial composition using either univariate or multivariate analyses (Table S1). Thus, we note
140 that all analyses have adjusted for potential confounders including age, sex, antibiotics, dietary
141 protein, and chronic conditions (diabetes and heart disease) unless stated otherwise. In some
142 analyses –that are noted below—we do also include vegetable and fruit dietary data.
143
144 **Social interactions and the human fecal microbiota** Human interactions were also associated
145 with differences in gut microbiota and diversity. Specifically, we found that individuals that were
146 cohabitating with a spouse or partner had more similar microbiota composition with their
147 cohabitating spouse/partner as well as higher diversity and richness than unmarried, non-
148 cohabitating individuals (unweighted UniFrac $P = 0.029$ Shannon $P = 0.005$, Chao $P = 0.011$, Fig.
149 2). Since all cohabitating pairs were male-female and sex was a strong determinant of the
150 microbiota in this study ($P < 0.001$, Table S2), increased diversity may be partially due to sustained
151 exchange of microorganisms between the sexes, though we were not able to test this given that
152 there were no same sex couples in these data. Increases in diversity seen here are consistent with
153 a previous cohabitation study in pigs [59] and may have implications for human health, as previous
154 work indicates that increased gut microbial diversity is associated with lower risks of irritable
155 bowel syndrome (IBS), Crohn's disease, ulcerative colitis, and other GI afflictions [60].
156 Social interactions with relatives and friends were stronger predictors of gut microbial
157 diversity in non-cohabiting individuals than cohabiting spouses/partners (unweighted UniFrac $P =$
158 0.0030, Shannon $P = 0.042$, Chao $P = 0.063$, Fig. S2) (Table S2). Here, social interactions were
159 defined as the sum of "How many times during the past four weeks have you gotten together with
160 relatives/friends?" The associations may have been weaker for cohabitating spouses due to their
161 higher microbial diversity; ecological theory supports that diverse communities are more resilient

162 and resistant to invasion by new species [61]. Thus, one explanation for these differential associations
163 is that the more diverse microbiotas of individuals already cohabitating with a spouse may not
164 have been as strongly influenced by increasing social interactions while the less diverse
165 microbiotas of those living alone were more strongly influenced by invasion of new species
166 through social exposures. It is also possible that cohabitating couples share the same friends and
167 socialize together with these friends. However, factors contributing to the resilience of the human
168 gut microbiota require further exploration to confirm this hypothesis.
169
170 **Spouses have more similar microbes than siblings and unrelated individuals** Previous studies
171 have established that the GI microbiota reaches an adult-like configuration by 3 to 5 years of age
172 [18,62,63] and that during adulthood, communities are stable on the time scale of years [19,20]. Thus,
173 microbial communities established in early life may persist and, aside from extreme perturbation,
174 remain stable across one's adult lifetime. However, our analyses comparing sibling, couple, and
175 unrelated pairs challenge the assumption that microbial communities established in early life will
176 be largely unperturbed in later life (for details, see METHODS, Statistical Analysis for spouse and
177 siblings). In fact, we find no evidence for a remaining influence of early life on the composition
178 of the gut microbiota among older adults. In this older cohort, spouses were more similar than
179 unrelated subjects (unweighted UniFrac P = 3.2E-5) or sibling pairs (unweighted UniFrac P =
180 0.033, Fig. 3). Further, the length of the cohabitating marital relationship was positively correlated
181 with similarity (unweighted UniFrac, P = 0.031) In contrast, siblings were no more similar than
182 unrelated pairs by any beta-diversity metric (P > 0.3, Fig. 3A, Fig. S3A, D, G) (Table S2). We also
183 found no evidence that the physical proximity of siblings—as measured by physical distance
184 between siblings—influenced gut microbial similarity. Thus, adult factors like marriage with

185 cohabitation (spouses) appear to have a greater influence on the adult gut microbiota than early-
186 life environment or genetics (siblings).

187 This is further supported by our findings that childhood farm status was not associated with
188 microbial richness (Chao $P = 0.342$) while working on a farm as an adult correlated with higher
189 richness (Chao $P = 0.005$). Farm-driven differences in the microbiota are of particular interest,
190 because adolescents that grew up on a farm have more diverse microbial communities [64] and
191 reduced risk of asthma and other atopic diseases both during childhood [65] and as adults [66]. Given
192 the results here, it appears that the microbially-driven protective effects of early farm exposures
193 are not due to the persistence of protective microorganisms acquired in early-life. Protection may,
194 instead, be conferred by immune development and training by early-life microbes as suggested
195 previously [67].

196 Our results are also in contrast with previous work showing that genetically related
197 individuals harbor more similar microbial communities than unrelated individuals, regardless of
198 current cohabitation [35,68–70]. However, these previous studies investigated children [68], young adults
199 [35,69], or a wide age range [70], and therefore, cumulative changes across a lifetime may not have
200 reached a level sufficient to overcome early-life factors impacting the microbiota. Additionally,
201 sibling pairs in other studies were twins [35,68–70], and many focused on monozygotic twins (same
202 sex and age) [35,69,70] as opposed to this study where siblings were often of opposite sexes (43%) and
203 ranged from less than a year to 18 years apart in age. Also, the unrelated group in this study may
204 have exhibited higher homogeneity than unrelated groups in other studies, because most grew up
205 in and/or currently live in the state of Wisconsin. Thus, compared to previous studies, siblings
206 were likely less similar and unrelated pairs more similar across our cohort. Furthermore, genetic
207 effects on the microbiota are often small [70] and detection may require a larger human cohort than

208 used here. Taken together, these factors may have contributed to the lack of significant differences
209 observed between sibling and unrelated groups even though average sibling beta-diversity was
210 intermediate between spouses and unrelated individuals.
211
212 **Increased microbial similarity, diversity, and richness in closer relationships** For both spouse
213 and sibling relationships, microbiota similarity was associated with self-reported relationship
214 closeness (unweighted UniFrac $P = 0.0079$). Closeness was measured by participant responses to
215 "How close are you and your current spouse/sibling?" on a scale of not at all (1) to very (4). Due
216 to the small sample sizes in the categories "Not very" (N=13) and "Not at all" (N=4), we combined
217 these two groups into "Not" close. Across spouses and siblings, individuals in very close
218 relationships harbored gut microbial communities more similar to their close social partners than
219 those in not very close relationships (Fig. 3B), though this relationship was not significant within
220 the spousal and sibling pair groups separately (Fig. 3C). Moreover, differences between spouses
221 and unrelated individuals, in terms of closeness (Fig. 2), as well as the enhanced diversity and
222 richness in cohabitating couples versus individuals living alone (Fig. 2) were driven by spouses
223 reporting very close relationships. This was in contrast to couples reporting only somewhat close
224 relationships as these pairs did not have higher gut microbiota similarity than unrelated pairs
225 (Table S3) nor did they display microbial diversity or richness different from non-cohabitating
226 individuals (Table S3). Importantly, the apparent impacts of relationship closeness do not appear
227 to be mediated by similarities in diet since overall dietary dissimilarity (Bray-Curtis and Jaccard)
228 did not significantly differ according to relationship closeness (ANOVA $P > 0.5$; Table S4). We
229 note that these included sensitivity tests that modeled diet based on the protein consumption, but
230 also overall diet that captured vegetable and fruit consumption.

231 While diet is often correlated with the GI microbiota [56], closeness points to the less well-
232 understood contributions of human interactions and shared behaviors. Close proximity and
233 frequent physical contact were correlated with microbiota similarity among primates with direct
234 microbial sharing between individuals contributing to similarity [22,23]. In this study, relationship
235 closeness may represent a summative measure of time spend together, physical affection, and other
236 human interactions with the potential to result in microbial sharing. Indeed, there is evidence that
237 the salivary microbiome influences the gut microbiome and the salivary microbiome may be
238 influenced by kissing [71,72]. In these data, this is supported by the fact that spouses had more
239 operational taxonomic units (OTUs, a proxy for microbial species) in common (30.4 ± 7.32%)
240 than siblings (26.4 ± 7.47%, t-test P = 4.39E-04) (Dataset S2). Also, when comparing the spouse
241 and sibling pair within a family represented in this dataset, a person tended to have more OTUs in
242 common with his or her very close spouse (25.4 ± 7.9%) than his or her very close sibling (22.2 ±
243 6.4%, N = 12 families, P = 0.074, Fig. 3D). This is also true when comparing very close spouses
244 (22.9 ± 5.8%) and somewhat close siblings within a family (20.6 ± 5.5%, N = 17 families, P =
245 0.027, Fig. 3E).
246
247 **Shared taxa with close human relationships**. In general, highly abundant genera and OTUs were
248 shared between many spouse and sibling pairs while less abundant shared taxa were specific to
249 one pair type and shared by a small number of pairs within that type (Dataset S3). OTUs that were
250 commonly found among spouses or siblings (> 50% of pairs) but rare in the unrelated dataset (<
251 70% individuals, < 49% unrelated pairs) may represent bacterial species easily shared by close
252 human interaction. These OTUs were predominately from the phylum Firmicutes (16 of 22 OTUs)
253 with representatives of families Lachnospiraceae and Ruminococcaceae (Dataset S4).

254 Interestingly, most of these potentially shared OTUs were from strictly anaerobic taxa, indicating
255 that persisting in an oxygen-rich environment in-between hosts may not be a limiting factor in very
256 close human relationships. Transmission, in these cases, could be mediated by direct contact
257 similar to mechanisms of vertical transmission from mother to child [73].

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

271 Overall, our findings indicate that in order to understand environmental influences on the
272 gut microbioata, we must now consider the many microbiotas with which this individual interacts.
273 Socialness with family and friends is associated with differences in the fecal microbiota. These
274 differences held even after accounting for dietary factors, though given this is the first study of its
275 kind, it will be critical for future work to validate this finding. Thus, it is possible that relationships
276 with others may influence the gut microbiota and consequent health outcomes, either through

direct microbial transfer or reinforcement of healthy microbiota behaviors. We further found not only that married couples had more similar gut microbiota but also that the microbiota of married individuals, compared to those living alone, has greater diversity and richness. Key to both of these findings, however, was that they were driven by individuals reporting that they were very close to their spouse as opposed to somewhat close. Close marriage relationships had a stronger influence than the shared genetic factors and early life environments among siblings. This finding is interesting, in part, because it parallels an extensive body of evidence demonstrating robust links between high quality marriages and morbidity and mortality. Future work could attempt to disentangle the mechanisms linking close relationships to microbial composition. For example, while we did not find evidence that shared diet was primarily responsible for these findings, we could not test precise frequencies of physical contact and intimacy as an alternative explanatory mechanism. Importantly, the types of physical contact and intimacy change over the life course, with sexual intimacy becoming far less frequent in later life, but other kinds of intimate physical contact remaining important. Regardless of the mediating mechanism, from a social and population health science perspective, decades of evidence that social relationships, especially close ones like marriage, influence morbidity and mortality make the central finding of significant interest. For example, even if future work finds a greater role for shared diets, it is still the social relationships that drive that shared diet. Overall, these results provide support for the gut microbiome as a possible mediating pathway between social relationships, especially marriage, and health and mortality. These findings, in the context of the robust body of evidence linking social relationships to human morbidity and mortality, provide fodder for further work examining the role of the gut microbiome as a possible biological mediator in these relationships [32]. Further microbiota work across time in a more diverse population should be undertaken with the many

300 longitudinal social science studies currently underway in an effort to increase our understanding
301 of the complex interactions between human behavior, the microbiota, and health.
302
303 **METHODS**
304 **Wisconsin Longitudinal Study (WLS)** WLS is based on a one-third sample of all 1957
305 Wisconsin high school graduates (N = 10,317) as well as selected siblings and spouses [54].
306 Graduates originally enrolled with an in-person questionnaire upon graduating high school in
307 1957, which was followed by data collection in 1964, 1975, 1992, 2004, and 2011. Siblings were
308 surveyed in 1977, 1994, 2005, and 2011; spouses were surveyed in 2004 or 2006. The content of
309 WLS surveys changed to reflect the participants' life course with an education focus in the initial
310 data collection, familial and career outcomes in young adulthood / midlife, and health, cognitive
311 functioning, psychological well-being, non-work activities, caregiving, bereavement, social
312 support, and end-of-life preparations in later rounds. WLS data collection was approved by the
313 Institutional Review Board (IRB) at the University of Wisconsin-Madison (2014-1066, 2015-
314 0955). Informed consent, the content and procedures of which were included in the IRB approval,
315 was obtained from participants. All methods were performed in accordance with relevant
316 guidelines and regulations.
317
318 **Study design** A total of 500 individuals were randomly drawn from the full WLS dataset
319 constrained based on the following: 1) participated in the 2011 interviews; 2) lived in one of 10
320 counties in Wisconsin that included both northern rural counties and southern more urban counties;
321 and 3) were part of a sibling pair. Individuals were removed from the study if they did not give
322 consent, their sample did not arrive for processing chilled, but not frozen, within 48 hrs of

323 collection, or their sample did not yield at least 10,000 sequences for analysis. This resulted in 408
324 individuals being included in this study.
325 An additional survey was administered at the time of fecal sampling, which detailed dietary
326 data from the prior three days, prescription/antibiotic use, current living situation, and additional
327 health information. This as well as selected data from the larger WLS study focused on health,
328 spouse/sibling relationships, and social interactions were used in this study (Text S1). Data,
329 documentation, and other materials are accessible at http://www.ssc.wisc.edu/wlsresearch/.
330 Access to the full dataset can be obtained through wls@ssc.wisc.edu.
331
332 **Sample collection** Stool samples were collected by participants in November 2014, January 2015,
333 or April 2015 following provided instructions (Text S2). Participants stored samples at ~4 °C in
334 their refrigerator or in a NanoCool box (Albuquerque, NM) with cooling cartridge and customized
335 foam insert, supplemented with a single ice pack. Interviewers picked-up samples from
336 participants within 24 hours of collection and shipped samples in fresh NanoCool boxes for arrival
337 at UW-Madison within 48 hours of collection. Upon arrival, an aliquot of feces was collected for
338 DNA extraction and immediately stored at -80°C until further processing. The use of WLS and
339 fecal microbiota data were approved by the Institutional Review Board at the University of
340 Wisconsin-Madison (2017-0600).
341
342 **DNA extraction** Genomic DNA was extracted from fecal aliquots using a bead-beating protocol
343 [45]. Briefly, feces (~100 mg) were re-suspended in a solution containing 500 µl of extraction buffer
344 [200 mM Tris (pH 8.0), 200 mM NaCL, 20 mM EDTA], 210 µl of 20% SDS, 500 µl
345 phenol:chloroform:isoamyl alcohol (pH 7.9, 25:24:1) and 500 µl of 0.1-mm diameter

346 zirconia/silica beads. Samples were mechanically disrupted using a bead beater (BioSpec Products,
347 Barlesville, OK; maximum setting for 3 min at room temperature), followed by centrifugation,
348 recovery of the aqueous phase, and precipitation with isopropanol. QIAquick 96-well PCR
349 Purification Kit (Qiagen, Germantown, MD) was used to remove contaminants. Isolated DNA was
350 eluted in 5 mM Tris/HCl (pH 8.5) and was stored at -80 °C until further use. We also note that we
351 used negative controls.
352
353 **Sequencing** PCR was performed using universal primers flanking the variable 4 (V4) region of
354 the bacterial 16S rRNA gene [82]. We used negative controls for each PCR reaction. PCR reactions
355 where the negative control yielded a product were not sequenced until the problem was solved.
356 Samples were processed all together, not in batches, in a random order (i.e., not clustered by
357 family). Additionally, unlike other specimens (e.g., saliva, skin), DNA contamination from
358 reagents is in general not a problem for fecal samples given the high DNA content of the sample
359 (10^{12} microbes/g of feces). In one reaction per sample, 10 - 50 ng DNA, 10 µM each primer, 12.5
360 µl 2X HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA), and water to 25 µl were
361 used. Cycling conditions were initial denaturation of 95 °C for 3 min followed by 25 cycles of 95
362 °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. PCR
363 products were purified with the QIAquick 96-well PCR Purification Kit (Qiagen, Germantown,
364 MD, USA). Samples were quantified by Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and
365 equimolar pooled. The pool plus 5% PhiX control DNA was sequenced through the U. of
366 Wisconsin-Madison Biotechnology Center with the MiSeq 2x250 v2 kit (Illumina, San Diego, CA,
367 USA) using custom sequencing primers [82]. All DNA sequences are available upon institutional
368 review board (IRB) or other ethics board approval through wls@ssc.wisc.edu.

369

370 **Sequence clean-up** All sequences were demultiplexed on the Illumina MiSeq. Sequence clean-
371 up and processing was performed with mothur v.1.36.1 [83] following a protocol similar to [82].
372 Briefly, paired-end sequences were combined into contigs with default parameters (match bonus
373 = 1, mismatch penalty = -1, gap penalty = -2, gap extend penalty = -1, insert quality ≥ 20,
374 mismatch quality difference ≥ 6). Poor-quality sequences, including those with ambiguous base
375 pairs, homopolymers greater than 8, or outside 200 – 500 bp in length, were discarded.
376 Sequences were then aligned to the SILVA 16S rRNA gene reference alignment database [84] and
377 trimmed to the V4 region. To reduce sequencing error, sequences with 2 or fewer differences
378 were pre-clustered. Chimera detection and removal were performed using UCHIME [85]. Final
379 sequences were then classified to the GreenGenes database [86]. Singletons were removed to
380 facilitate downstream analyses. All sequences were grouped into 98% operational taxonomic
381 units (OTUs) by uncorrected pairwise distances and average neighbor clustering in mothur.
382 Clustering performed on uncorrected pairwise distances revealed no differences in clusters at 97
383 vs 98% similarity. Therefore, the stricter cutoff was reported Coverage was assessed by Good's
384 coverage, and then samples were normalized to whole number counts by percent
385 relative abundance to approximately 10,000 sequences per sample (9,914 - 10,061 after
386 rounding.

387

388 **Statistical analysis for graduates** Graduates were assessed separately from siblings and spouses
389 to avoid potential interactions, and the graduate subset was not significantly different from other
390 groups (PERMANOVA *P* Bray-Curtis $P = 0.56$, Jaccard $P = 0.57$, weighted UniFrac $P = 0.33$,
391 unweighted UniFrac $P = 0.24$). Alpha-diversity was assessed with Shannon's diversity and Chao's

392 richness calculated in mothur. Differences in alpha-metrics were assessed in R v3.3.2 [87] by linear
393 regression with the Benjamini-Hochberg correction for multiple comparisons across each metric.
394 Microbial beta-diversity was assessed for Bray-Curtis, Jaccard, weighted, and unweighted
395 UniFrac metrics with results shown for unweighted UniFrac unless otherwise noted. Dietary beta-
396 diversity was assessed for Bray-Curtis and Jaccard metrics as well as corresponding nMDS axes
397 calculated from habitual intake of specific sources of protein (N = 4), vegetables (N = 76), and
398 fruits (N = 24) expressed as times consumed per week (protein), proportions of total types (all),
399 and presence/absence of individual types (all). Differences in beta-diversity were tested with
400 permutational analysis of variance (PERMANOVA, adonis) in the vegan package [88] with the
401 Benjamini-Hochberg correction for multiple comparisons across each metric and a maximum of
402 5000 permutations. All variables were modeled using independent, univariate tests and dietary
403 variables were additionally modeled using multivariate tests of all components (protein,
404 vegetables, fruits). Co-variance of microbial and dietary beta metrics was measured using Mantel's
405 test. The factors that associated with the microbiome in univariate models (*i.e.* age, sex, antibiotics,
406 dietary protein, high blood sugar, and heart disease [57]) were adjusted for in regression models as
407 potential confounders. Beta-diversity was visualized by non-metric multidimensional scaling
408 (nMDS) plots with arrows from significant variables (PERMANOVA) fitted to the ordination
409 using maximum correlation (envfit, vegan). All tests were assessed at significance $P < 0.05$ and
410 trends $0.05 < P < 0.1$.
411
412 **Statistical analysis for spouses and siblings**
413 For the spouse and sibling similarity analysis, the unit of the observation is the pair (*i.e.* spouse,
414 sibling, or unrelated pair defined below) and the variables used in the analysis are distance in

individual measurements between the two members of the pair. Specifically, beta-diversity metrics were used to quantify the distance in microbial and overall diet whereas absolute difference were calculated to quantify the distance in all the other variables (*e.g.* age, sex, dietary protein). We sampled unrelated pairs from the data in order to compare the spouse or sibling pair with unrelated pairs. In particular, the unrelated individuals cannot be siblings, spouses, or in-laws, and each unrelated pair will match the corresponding spouse or sibling pair in sex and antibiotics usage. Beta-diversity distances were compared among spouse, sibling, and unrelated pairs using linear regression while adjusting for the distance in age, sex, dietary protein, health conditions (if available). P-values were averaged across 1000 rounds of unrelated pair sampling. For closeness analysis, we removed age and sex from the model because the two variables are highly correlated with pair type (*i.e.* sibling/spouse pair can be accurately classified using the difference of the age or sex between the two members of the pair). For comparing OTU sharing among spouse and sibling within a family, we used mixed-effect models to account for family clustering. All tests were assessed at significance $P < 0.05$ and trends $0.05 < P < 0.1$.

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627 **AUTHOR CONTRIBUTIONS**

628 FER and PH designed research. JHK, RLK, and TS completed sample processing and sequencing.
629 AP contributed to the conceptual and analytic approach to conducting the analyses. KADM, ZZT,
630 and GC analyzed the data. KADM, ZZT, GC, FER, and PH contributed to the final manuscript.

631 Alberto Palloni[5]

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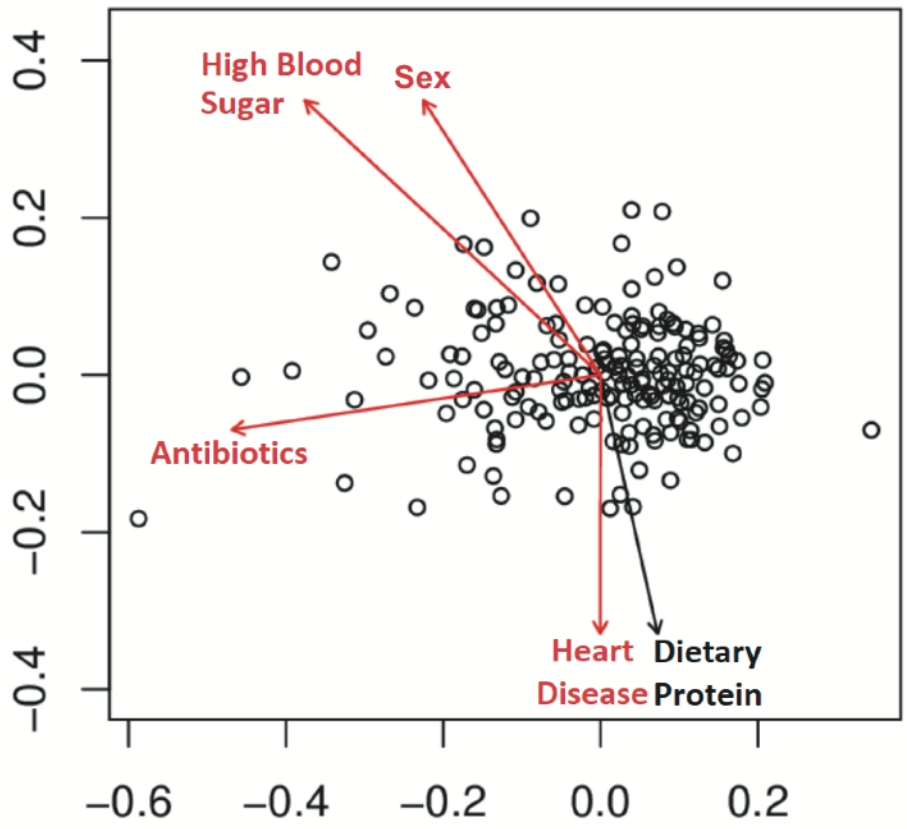
645 interests that might be perceived to influence the results and/or discussion reported in this paper.

646 **FIGURES**

647

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



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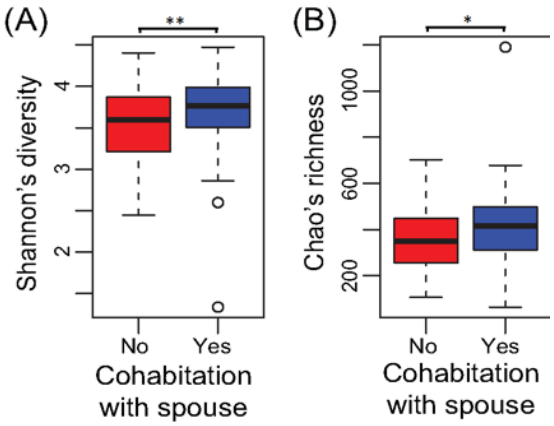
658 **Figure 2.** Cohabitation is associated with increased alpha-diversity. Boxplots of (A) Shannon's
659 diversity and (B) Chao's richness of graduates that are (blue) or are not (red) cohabitating with a
660 spouse or partner. All spouses/partners were cohabitating while all non-cohabitating individuals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



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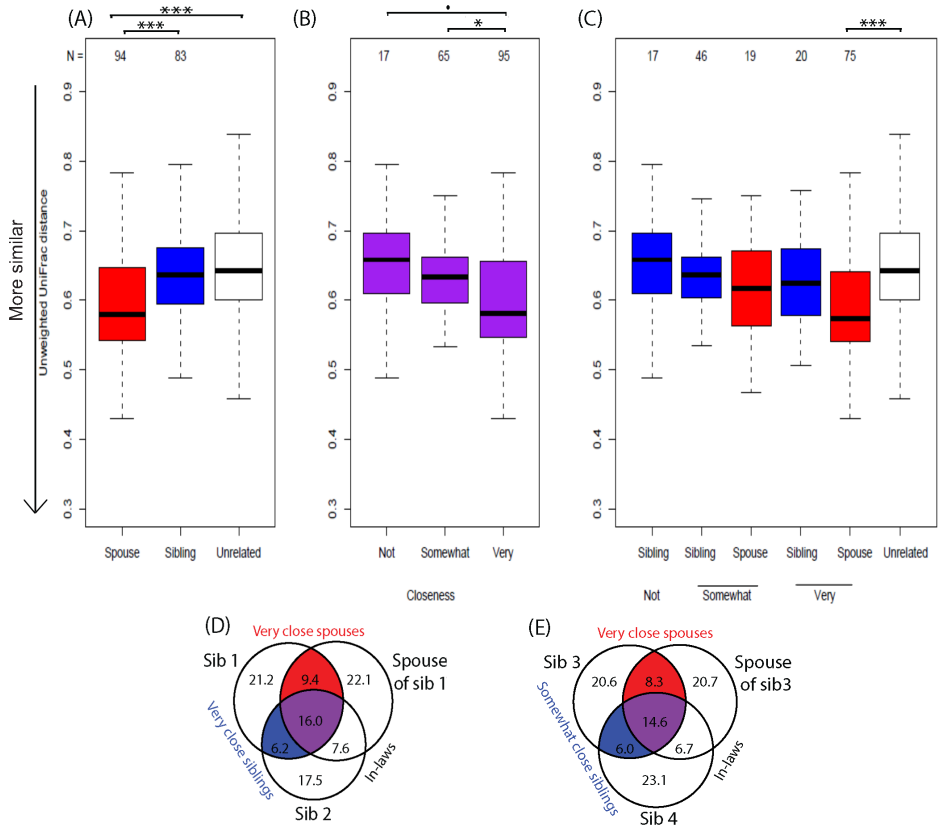
676 **Figure 3.** Microbial sharing in spouse and sibling relationships. Unweighted UniFrac distances of
677 (A) spouse, sibling, and unrelated pairs, (B) spouses and siblings grouped by relationship
678 closeness, and (C) spouses and siblings separated by relationship closeness. Groups (A) were
679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



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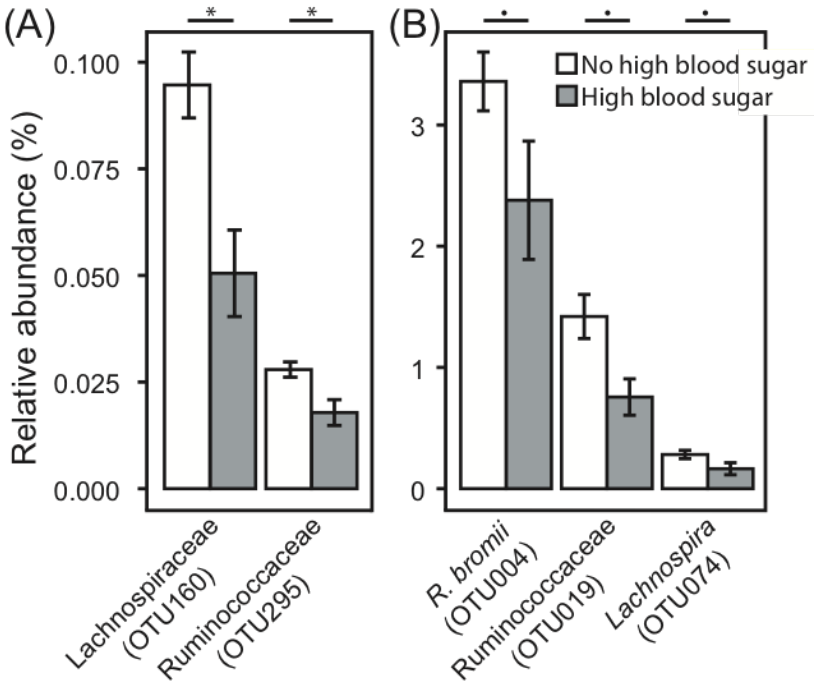
688 **Figure 4.** Percent relative abundance of OTUs that are commonly shared between spouses and
689 that differed between those with (grey) and without (white) high blood sugar. (A) Low abundance
690 and (B) more highly abundant OTUs. Means with standard error bars are shown. Kruskal-Wallis
691 FDR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



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