Interactions between social groups of colobus monkeys (*Colobus vellerosus*) explain similarities in their gut microbiomes

Abstract

The gut microbiome is structured by social groups in a variety of host taxa. Whether this pattern is driven by relatedness, similar diets, or shared social environments is under debate because few studies have had access to the data necessary to disentangle these factors in wild populations. We investigated whether diet, relatedness, or the 1-meter proximity network best explains differences in the gut microbiome among 45 female colobus monkeys in 8 social groups residing at Boabeng-Fiema, Ghana. We combined demographic and behavioural data collected May-August 2007 and October 2008-April 2009 with 16S rRNA sequencing of faecal samples collected during the latter part of each observation period. Social group identity explained a large percentage of the variation in gut microbiome beta-diversity. When comparing the predictive power of dietary dissimilarity, relatedness, and connectedness in the 1-meter proximity network, the models with social connectedness received the strongest support, even in our analyses that excluded within-group dyads. This novel finding indicates that microbes may be transmitted during intergroup encounters, which could occur either indirectly via shared environments or directly via social contact. Lastly, some of the gut microbial taxa that appear to be transmitted via 1-meter proximity are associated with digestion of plant material, but further research is needed to investigate whether this type of gut microbe transmission yields health benefits, which could

provide an incentive for the formation and maintenance of social bonds within and between social groups.

Key words: 16S rRNA gene, between-group encounters, colobines, diet, gut microbiome, microbe transmission, relatedness, social networks, social transmission, host-microbe

Introduction

The gut microbiome consists of thousands of species that affects its host's nutritional status, immune function, and behavior (McFall-Ngai et al., 2013). It is associated with parasite resistance and stress response of hosts in the wild (Koch & Schmid-Hempel, 2011; Vlčková et al., 2018) and with obesity in captive settings (Turnbaugh et al., 2006). Because of these potential health consequences, it is important to investigate the acquisition and maintenance of the gut microbiome (Amato, 2016; Archie & Tung, 2015).

The gut microbiome of individuals or social groups become more distinct with geographic distance (Barelli et al., 2015; Grieneisen et al., 2019; Hansen et al., 2019; Hird, Carstens, Cardiff, Dittmann, & Brumfield, 2014; Lankau, Hong, & Mackie, 2012; Phillips et al., 2012), and the microbiome is structured by social group or family co-residency in a variety of host taxa, such as humans (Lax et al., 2014; Song et al., 2013; Yatsunenko et al., 2012), non-human primates (Amato et al., 2017; Degnan et al., 2012; Goodfellow et al., 2019; Orkin, Webb, & Melin, 2019; Springer et al., 2017; Tung et al., 2015), carnivores (Leclaire, Nielsen, & Drea, 2014; Theis et al., 2013), birds (White et al., 2010), and insects

(Anderson et al., 2012; Koch & Schmid-Hempel, 2011). For example, the gut microbiome of immigrant male baboons *(Papio cynocephalus)* converge over time with that of their new group members (Grieneisen, Livermore, Alberts, Tung, & Archie, 2017), and the gut microbiomes of colobus monkeys (*Colobus vellerosus*) diverged over the course of nine months after a social group fissioned into two daughter groups (Goodfellow et al., 2019).

The divergence in gut microbiomes with home range separation could potentially be due to dietary differences, lower degrees of relatedness, or lack of shared social environments (Archie & Tung, 2015; Björk, Dasari, Grieneisen, & Archie, 2019). Diet is suggested to be one of the most important factors affecting the gut microbiome (Voreades, Kozil, & Weir, 2014). Gut microbial composition fluctuates within hosts with seasonal or experimental dietary changes (Davenport et al., 2014; David et al., 2013; Hicks et al., 2018; Leamy et al., 2014; Mallott, Amato, Garber, & Malhi, 2018; Maurice et al., 2015; Michl et al., 2019; Orkin, Campos, et al., 2019), and dietary similarities may explain whether social groups have distinct gut microbiomes (Orkin, Webb, et al., 2019). However, this pattern could also reflect the genetic similarity of hosts in societies where at least some closely related individuals remain together in their natal group. When this is the case, closely related group members are expected to have more similar gut microbiomes than non-group members with lower degree of relatedness, because the host's genetic makeup affects microbe colonization (Opstal & Bordenstein, 2015; Spor, Koren, & Ley, 2011) and a number of genomic regions are associated with gut microbial composition in rodents (Bonder et al., 2016; Leamy et al., 2014). This may explain why closely related individuals have more similar gut microbiomes than unrelated individuals in some studies of humans and captive rodents

(Faith et al., 2013; Kovacs et al., 2011; Ley et al., 2005). In contrast, genetic differentiation between baboon populations was a poor predictor of their gut microbiome (Grieneisen et al., 2019), and relatedness did not have a significant effect on the gut microbiome in some studies of humans (Rothschild et al., 2018), non-human primates (Moeller et al., 2016) and carnivores (Leclaire et al., 2014). Moeller and colleagues (2016) suggest that this may be due to an overriding effect of transmission among unrelated social partners. Indirect social contact via shared environments, such as touching common surfaces, may facilitate microbiome transmission within households (i.e., indirect social transmission) (Lax et al., 2014). Direct social contact such as grooming or sitting in body contact further increases microbiome transmission (i.e., direct social transmission) between close social partners within social groups of monkeys (*Alouatta pigra*: Amato et al., 2017; *Papio cynocephalus*: Tung et al., 2015; Grieneisen et al., 2017) and lemurs *(Eulemur rubriventer:* Raulo et al., 2017*)*. Gut microbiomes are also more similar among socially connected than disconnected siblings and married couples (Dill-McFarland et al., 2019). In contrast, social connectedness between non-group members did not predict gut microbiome similarity in sifakas (*Propithecus verreauxi*) (Perofsky et al., 2017). Even if intergroup encoutners promote the transmission of microbes, there may not be an association between intergroup interactiowns and gut microbiome similarity if groups rarely interact at close distances. Taken together, these studies indicate that gut microbes are transmitted via social interactions within social groups, while it is unclear whether this is the case for social interactions between social groups.

To investigate whether the pattern of increasing between-individual differences in the gut microbiome (i.e., beta-diversity) with home range separation is best explained by lower dietary overlap,

relatedness, or social connectedness, we focus on the black-and-white colobus monkeys (*Colobus vellerosus*) at Boabeng-Fiema, Ghana. This is one of several rare species of arboreal leaf-eating monkeys distributed across the forested regions of the African tropics, and it is closely related to guerezas (*Colobus guereza*) and western black-and-white colobus (*Colobus polykomos*) (Ting, 2008). At Boabeng-Fiema, all colobus social groups utilize a highly folivorous diet, but the most important food species differ between social groups (Saj & Sicotte, 2007; Teichroeb & Sicotte, 2009). More seeds and fruits are available during the dry season, during which they eat up to 43% of these food items (Teichroeb & Sicotte, 2017). To break down hard-to-digest items in their primarily folivorous diet (Saj & Sicotte, 2007; Teichroeb & Sicotte, 2009), they rely on behavioural traits, physiological traits, and their gut microbiome (Amato et al., 2016; Lambert, 1998). Possibly due to constraints imposed by their highly folivorous diet, colobus monkeys spend a low percentage of their time engaging in direct social activities such as grooming (Teichroeb, Saj, Paterson, & Sicotte, 2003). Female colobus spend on average 3% of their time within 1 meter and 0.1% of their time grooming each female group member (Wikberg, Ting, & Sicotte, 2014b). However, females still form preferred friendships, which are only occassionally based on kinship and never based on their relatively weakly expressed dominance hierarchies (Wikberg, Teichroeb, Bădescu, & Sicotte, 2013; Wikberg, Ting, & Sicotte, 2014a; Wikberg et al., 2014b; Wikberg, Ting, & Sicotte, 2015). Instead, females prefer to affiliate with females with similar immigration status (Wikberg et al., 2014b, 2014a) in this population where all males and half of the females disperse (Sicotte et al., 2017; Teichroeb, Wikberg, & Sicotte, 2009, 2011; Wikberg, Sicotte, Campos, & Ting, 2012). This flexible female dispersal pattern results in social groups with different female kin composition and some close

100 maternal female kin residing in different social groups (Wikberg et al., 2012). Neighbouring social groups
101 encounter each other in the large zones of home range overlap on an almost daily basis. During these
102 encounters, social groups sometimes chase each other away from food trees, while at other times, they
103 engage in affiliative or sexual between-group interactions (Sicotte & MacIntosh, 2004; Teichroeb &
104 Sicotte, 2017).

105 The frequent between-group interactions coupled with variation in diet and relatedness within
106 and between social groups makes this a good study population to investigate whether the pattern of
107 increasing gut microbial beta-diversity with home range separation is best explained by lower degrees of
108 dietary similarity, relatedness, or social connectedness. We take a cross-sectional approach using
109 observational and genetic data from eight social groups to first test whether the gut microbiome was
110 structured by social groups. We predicted gut microbiome beta-diversity to be structured by social
111 groups and to increase with home range separation. We then evaluated which factors explained gut
112 microbiome beta-diversity between females across different social groups. We expected gut
113 microbiome beta-diversity to decrease with dietary similarity and relatedness and increase with distance
114 in the 1-meter proximity network. Finally, the significant predictor from the analyses above (social
115 connectedness) was used in a subsequent population-level analysis of Operational Taxonomic Unit
116 (OTU) abundance to determine which microbial taxa may be socially transmitted. Our definition of social
117 transmission includes both direct social transmission via physical contact and indirect social transmission
118 via shared substrates (e.g., Perofsky et al., 2017), and we will not attempt here to tease these two social
119 transmission routes. Males and females of all age-classes were used to create social networks, but the

gut microbiome data are only available for adult females. Therefore, our analyses of beta-diversity focus on adult females.

Methods

Behavioral data collection

Demographic data have been collected since 2000 from the black-and-white colobus monkeys (*Colobus vellerosus*) at Boabeng-Fiema, Ghana. In this study, we also use behavioral and ecological data as well as DNA samples from eight social groups (Fig. A1) collected during two study periods: the rainy season May-August 2007 and the pre-dry and dry seasons October 2008 - April 2009 (Table A2). During this time period, the study groups contained 3-9 adult (i.e., parous) females (Table A2), 1-4 adult males, and 8-24 immatures. Our research adheres to ASAB/ABS Guidelines for the Use of Animals in Research, the laws of Ghana, and data collection was approved by the Boabeng-Fiema Monkey Sanctuary's management committee, Ghana Wildlife Division, and the University of Calgary's Animal Care Committee (BI 2006-28, BI 2009-25).

We recorded the social group's location every hour using a map with trails, roads, villages, and large trees (>40 cm DBH) in order to determine home ranges (Fig. A1). During 10-minute focal samples (Altmann, 1974) of adult females, we continuously recorded all social behaviors (including the identity of the interactant and the duration of the behavior) and plant species and part (i.e., mature leaf, young leaf, flower, fruit, seed, or other) for each ingested food item. Females fed on a total of 210 food item-plant species combinations, and to assess dietary differences, we calculated Sørensen dissimilarity

140 indices using ingested plant parts and plant species during focal samples. We choose this diversity index
141 because it only takes the presence or absence of an ingested food item into account, which we have a
142 robust estimate of using the focal data. The Sørensen dissimilarity indices in our data set had a high
143 median value of 0.83 and it was lower within than between social groups (Fig. A2).

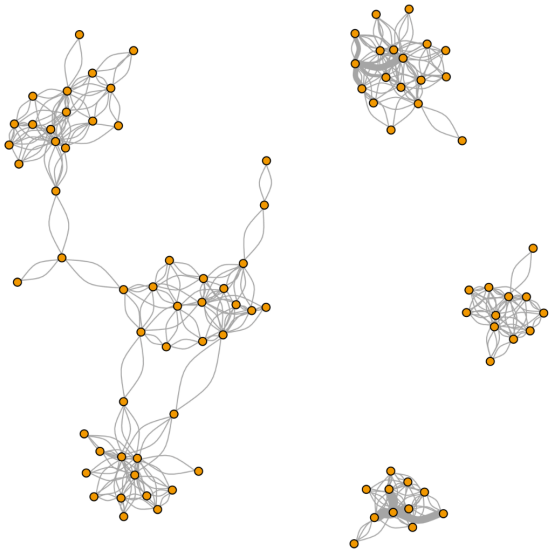
144 We observed 61 and 285 between-group encounters (i.e., two social groups located within 50
145 meters of each other) during the first and second data collection period respectively. Of these
146 encounters, 53% lacked female aggression and 35% lacked male aggression. Because close proximity
147 between individuals of different social groups are rare and unlikely to be recorded during focal sampling,
148 we recorded approaches to 1 meter *ad libitum* (Altmann, 1974). Some of these approaches only led to
149 brief close proximity while others led to prolonged contact like copulations, grooming, and play. We
150 created an undirected proximity network based on the presence and absence of approaches to 1 meter
151 between all individuals (N = 177 adult females, adult males, and immatures) present in the eight study
152 groups. We used the software UCINET (Borgatti, Everett, & Freeman, 2002) to compute inverse shortest
153 path length (i.e., Geodesic distance) in the 1-meter proximity network (hereafter referred to as social
154 connectedness): 1/[the number of steps (i.e., recorded interaction ties) in the shortest path from one
155 individual to another]. Social group members were in 1-meter proximity with each other (i.e., an inverse
156 path length of 1) or separated by two to three partners (i.e., an inverse path length of 0.5 and 0.33) (Fig.
157 A2). The inverse path length for males and females belonging to different social groups ranged from 0 to
158 1 (Fig. 1; Fig. A2). The seemingly unconnected individuals in the 2007 data set were most likely
159 unconnected because we only had access to data collected from a 3-month period. These individuals

were connected and separated with up to eight steps in the 2008-2009 network, which was based on six months of data.

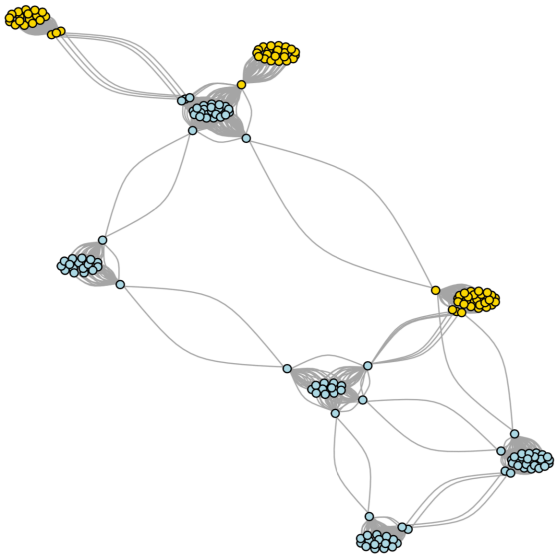
Genetic data collection

We collected faecal samples June-August 2007 and January-April 2009. Immediately after a female defecated, we collected approximatey 1g of feces and dissolved it in 6ml RNAlater. The samples were stored in a fridge at the field site until the end of the field season when they were transported to the Ting lab and stored in a -20-degree C freezer. Note that we lack information on soil type, which was driving between-site differences in the gut microbiomes in a large-scale study of terrestrial baboons (Grieneisen et al., 2019). However, our samples were collected from arboreal primates within a small study area, and sampling site does not have a significant effect on beta-diversity in our study population (Goodfellow et al., 2019).

(a) 2007

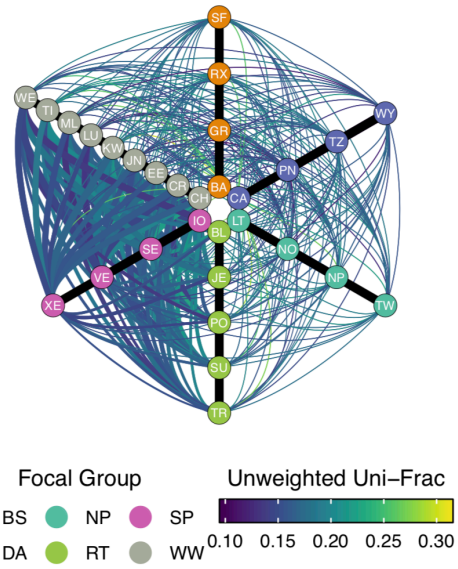


(a) 2009

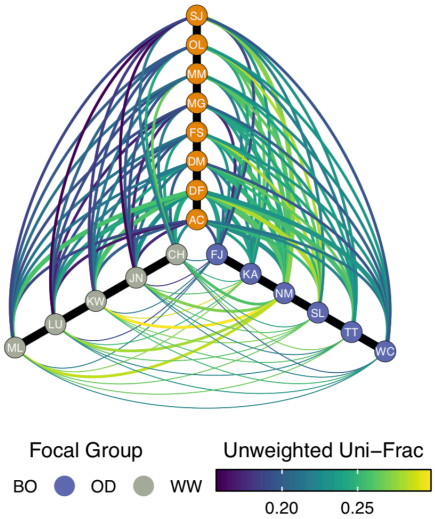


173

(b) 2007



(b) 2009



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175

176 **Figure 1.** Social networks for: **a)** the entire population where each group member is depicted as a node
177 in yellow (group used for behavioural analyses) or blue (group not used for behavioural analyses) and
178 between-group dyads observed in 1-meter proximity are connected with lines; **b)** females included in
179 the behavioural analyses with lines connecting between-group dyads (i.e., nodes of different color)
180 where color represents gut microbiome beta-diversity (i.e., unweighted Unifrac distances) ranging from
181 similar (dark) to dissimilar (light) and thickness indicates social connectedness ranging from strongly
182 connected (thick) to more disconnected (thin). The black lines connect group members and are not
183 weighted based on beta-diversity or social connectedness.
184
185 We extracted DNA from the samples and genotyped the extracts at 17 short tandem repeat loci
186 (STR) as previously described (Wikberg et al., 2012). To make sure that the samples used in the
187 relatedness and gut microbiome analyses were collected from the correct individual, we compared the
188 STR genotypes obtained from these samples with a second sample collected from the same individual at
189 a different time. We calculated dyadic estimated relatedness values (*R*) in MLRelate (Kalinowski,
190 Wagner, & Taper, 2006) because this method provided the most accurate relatedness estimates in our
191 study population (Wikberg et al., 2012). We used *R*-values calculated from STR loci rather than
192 theoretical relatedness (*r*) calculated from pedigrees, because *R*-values predict kinship relatively
193 accurately in our study population (Wikberg et al., 2014a) and they are more accurate than *r* in studies
194 such as ours with limited access to pedigrees (Forstmeier, Schielzeth, Mueller, Ellegren, & Kempenaers,
195 2012; Robinson, Simmons, & Kennington, 2013). The median female relatedness was low both within

196 and between social groups, but there were at least some closely related females residing in the same
197 social groups (Fig. A2).
198 For generating the gut microbial data, we conducted fresh DNA extracts from 61 previously
199 genotyped samples from 45 females (Table A2) using the QIAamp DNA Stool Mini Kit with a modified
200 protocol. More details regarding the extraction protocol are presented in the Appendix and in
201 Goodfellow et al. (2019). The V4 hypervariable region of the bacterial 16S ribosomal RNA gene was
202 amplified and libraries were prepared using the 515F and 806R primers containing 5' Illumina adapter
203 tails and dual indexing barcodes, and libraries were sequenced as part of a 150bp paired-end sequencing
204 run on the Illumina NextSeq platform following Goodfellow et al. (2019). We obtained a mean read
205 depth of 127,628 per sample (range: 86,924-166,438). Then, we used a custom pipeline
206 (https://github.com/kstagaman/Process_16S) for quality filtering and assembly (see Appendix). We
207 performed *de novo* OTU picking in UCLUST (Edgar, 2010), and sequences with 97% overlap were defined
208 as belonging to the same bacterial Operational Taxonomic Unit (OTU). After this processing, we had a
209 total of 2,597 OTUs and an average of 89,483 reads per sample (range: 59,817-120,119). To further
210 guard against sequencing errors, we filtered out OTU's with a frequency lower than 0.00005 as
211 recommended (Bokulich et al., 2012). After filtering, the 2007 data set contained 450 OTUs and the
212 2009 data set contained 396 OTUs. The mean read depth was 88,346 (range: 59,005 – 118,633). We did
213 not rarefy the data set to an even read depth, because it is recommended against (McMurdie & Holmes,
214 2014). First, rarefying leads to increased false positives and decreased true positives, especially in data
215 sets with read depths comparable to ours (Pereira et al., 2018). Second, unrarefied counts are

216 particularly accurate when using our measure of beta-diversity—weighted UniFrac distances (McMurdie
217 & Holmes, 2014).
218 We initially calculated four different measures of gut microbiome beta-diversity (Sørensen
219 dissimilarity index, Bray-Curtis dissimilarity index, unweighted UniFrac distances, and weighted UniFrac
220 distances) in the R package vegan (Oksanen et al., 2017). Because the two presence/absence indices
221 were strongly correlated with each other (Sorenson dissimilarity indices and unweighted UniFrac
222 distances: Mantel r = 0.93, p = 0.001) as were the two abundance indices (Bray-Curtis dissimilarity
223 indices and weighted UniFrac distances: Mantel r = 0.77, p = 0.001), in our analyses, we only used the
224 one presence/absence index (unweighted UniFrac distances) and the one abundance index (weighted
225 UniFrac distances) that take phylogenetic relationships of OTUs into account.
226
227 *Data analyses*
228 We combined the 2007 and 2009 data sets and included study year (aka season) and individual ID as
229 predictor variables whenever possible (i.e., permutational multivariate analysis of variance and linear
230 mixed models) while we had to create squared interaction matrices for each study year separately when
231 using matrix correlations (i.e., Mantel tests and Moran's test for autospatial correlations). We only used
232 the full data set (N = 61 samples from 45 females) for the initial analysis regarding the effect of social
233 group identity. All subsequent analyses examined the effects of behavioural variables on beta-diversity
234 in a subset (N = 49 samples from 42 unique females) from which we removed: 1) duplicate samples from
235 the same year and same female; 2) one adult female with incomplete dietary information, and 3) social

236 groups from which the majority of females remained unsampled to make sure we had a representative
237 sample of social connectedness from each social group.

238 The initial analysis investigated the effects of season, social group, individual identity, and read
239 depth on beta-diversity of all dyads in the full data set (N = 61 samples from 45 females in 2007 and
240 2009) using permutational multivariate analysis of variance (PERMANOVA) with 10,000 permutations
241 using the adonis function in the R package vegan (Oksanen et al., 2017). The terms were added
242 sequentially in the order listed above.

243 We used non-parametric Mantel correlations implemented in the R package vegan (Oksanen et
244 al., 2017) to investigate whether the two measures of gut microbiome beta-diversity were correlated
245 with home range separation (0 = same social group and home range; 1 = different social groups but
246 adjacent home ranges, 2 = different social groups and non-adjacent home ranges) using beta-diversity
247 indices from 30 samples from unique females in 6 social groups in 2007 and 19 samples from unique
248 females in 3 social groups in 2009. We used beta-diversity indices of all dyads, but analysed the two
249 years separately.

250 To investigate which combination of dyadic traits predicted gut microbiome beta-diversity
251 between females, we created generalized linear mixed models (GLMMs) with the outcome variable gut
252 microbiome beta-diversity using the beta family function in the package glmmTMB (Magnusson et al.,
253 2019) in R (R Core Team, 2018). Again, we used 30 samples from unique females in 6 social groups in
254 2007 and 19 samples from unique females in 3 social groups in 2009. We created a null model that did
255 not contain any fixed effects, alternative models with one fixed effect that represented one of the

256 hypotheses outlined in the introduction (dietary dissimilarities, *R*-values, or social connectedness), and a
257 full model with all three predictor variables. We included data collection year as a fixed effect in all
258 alternative models because the two sampling years occurred in different seasons and several other
259 studies show strong seasonal shifts in gut microbiome composition (Amato et al., 2015; Hicks et al.,
260 2018; Orkin, Campos, et al., 2019; Smits et al., 2017; Springer et al., 2017). All numerical predictor
261 variables were centered and scaled (Schielzeth, 2010). We included social group and focal identities as
262 random effects in all GLMMs, including the null models. We did not have any issues with collinearity
263 based on low Variance Inflation Factors for the full models (all VIF < 1.43). We evaluated the support for
264 each model using Akaike Information Criterion (AIC) (Akaike, 1974), and this approach allowed us to
265 determine which hypotheses (diet, relatedness, or social connectedness) was best supported by our
266 data (Burnham & Anderson, 2002). Because several models received similar support, we took model
267 selection uncertainly into account by averaging coefficients across models (Burnham & Anderson, 2002)
268 using the R package MUMIN (Barton, 2013). In the first set of analyses, we included dyads that resided
269 in the same social group and dyads that resided in different social groups. To make sure that the effect
270 of social connectedness was not driven by the close social bonds within social groups, we repeated the
271 analyses with between-group dyads only.

272 To infer which of the gut microbial taxa may be transmitted via close proximity, which was a
273 better predictor of beta-diversity than diet and relatedness (see Results), we investigated whether the
274 abundance of each OTU was correlated with Geodesic distance in the 1-meter approach network using
275 Moran's test for autospatial correlations implemented in the package ape (Paradis, Claude, & Strimmer,

276 2004). We included within-group and between-group dyads in this analysis (N = 342 dyads). We counted
277 the number of OTUs in each phylum (or family) that were socially structured based on the autospatial
278 correlation results. We conducted hypergeometric tests to investigate whether this number was higher
279 than expected by chance based on the total number of OTUs in the phylum (or family) using the phyper
280 function implemented in R. In all analyses of taxonomic differences, we used the 10% false discovery
281 rate to correct p-values for multiple testing (sensu Tung et al., 2015). The gut microbial taxa we
282 expected to be shaped by sociality are listed in the Table A1 (Amato et al., 2017; Goodfellow et al., 2019;
283 Tung et al., 2015).
284

285 Results

286 *Factors predicting gut microbiome beta-diversity*

287 We investigated the relative effects of season, social group, individual ID, and read depth in the full data
288 set (PERMANOVA: N = 61 samples collected 2007-2009). Of the observed variation in the taxonomic
289 composition of the gut microbiome (i.e., beta-diversity), individual identity explained the largest
290 percentage (54-55% depending on which beta-diversity index was used as outcome variable), social
291 group identity explained a more moderate percentage (19-28%), while year explained much smaller
292 percentage (8-12%) (Table 1). Read depth did not have a significant effect on beta-diversity (Table 1).

Beta-diversity index	**Factor**	**Df**	**Sums of squares**	**Mean squares**	**F**	**R^2**	**P**
Unweighted UniFrac	Season	1	0.103	0.103	12.325	0.084	<0.001
	Group	7	0.347	0.050	5.943	0.282	<0.001
	ID	39	0.663	0.017	2.035	0.538	<0.001
	Read depth	1	0.010	0.010	1.185	0.008	0.249

Weighted UniFrac	Season	1	0.136	0.136	12.277	0.118	<0.001
	Group	7	0.219	0.031	2.812	0.189	<0.001
	ID	39	0.639	0.016	1.477	0.553	<0.001
	Read depth	1	0.018	0.018	1.637	0.016	0.104

Table 1. Results from the PERMANOVA with factors added sequentially in the ordered listed in the table.

Gut microbiome beta-diversity and home range separation were correlated in the 2007 data set (N = 870 dyads in 6 social groups, Mantel tests: unweighted UniFrac distance: r = 0.22, P = 0.002; weighted UniFrac distance: r = 0.10, P = 0.049) and in the 2009 data set (N = 342 dyads in 3 social groups, Mantel tests: unweighted UniFrac distance: r = 0.36, P = 0.005; weighted UniFrac distance: r = 0.20, P = 0.024), meaning that females residing farther from each other had less similar gut microbiomes. This pattern can potentially be explained by group members having more similar diets, higher relatedness, or stronger social connectedness than non-group members (Fig. A2).

We created several competing generalized linear mixed models to investigate which of the three hypotheses best explained increasing beta-diversity with home range separation: dietary dissimilarity, relatedness, or social connectedness, controlling for data collection year. In our data set with both within-group and between-group dyads (N = 1,212 dyads in 2007-2009), the full models and the models with social connectedness received the greatest support (Table 2). Social connectedness predicted gut microbiome beta-diversity, and females located further apart in the social network had less similar gut microbiomes (Figs. 1-2). Year also predicted gut microbiome beta-diversity (Fig. 2), and females had more similar gut microbiomes during the rainy season of 2007 than the dry season of 2009. In contrast, diet and relatedness did not have significant effects on gut microbiome beta-diversity (Fig. 2).

Outcome variable	Fixed effect	AIC	Delta	Weight
Unweighted UniFrac	Season + Proximity	-5247.30	0.00	0.73
	Season + Diet + Relatedness + Proximity	-5245.28	2.02	0.27
	Season + Diet	-5103.63	143.67	0.00
	Season + Relatedness	-5060.36	186.94	0.00
	-	-4940.73	306.58	0.00
Weighted UniFrac	Season + Proximity	-4684.20	0.00	0.56
	Season + Relatedness	-4683.69	0.51	0.44
	Season + Diet	-4658.94	25.25	0.00
	Season + Diet + Relatedness + Proximity	-4657.85	26.35	0.00
	-	-4614.36	69.84	0.00
Unweighted UniFrac	Season + Proximity	-4307.62	0.00	0.76
	Season + Diet	-4303.92	3.71	0.12
	Season + Relatedness	-4302.67	4.95	0.06
	Season + Diet + Relatedness + Proximity	-4302.44	5.19	0.06
	-	-4119.55	188.07	0.00
Weighted UniFrac	Season + Proximity	-3770.21	0.00	0.64
	Season + Diet	-3768.91	1.30	0.33
	Season + Relatedness	-3762.64	7.58	0.01
	Season + Diet + Relatedness + Proximity	-3761.96	8.25	0.01
	-	-3713.73	56.48	0.00

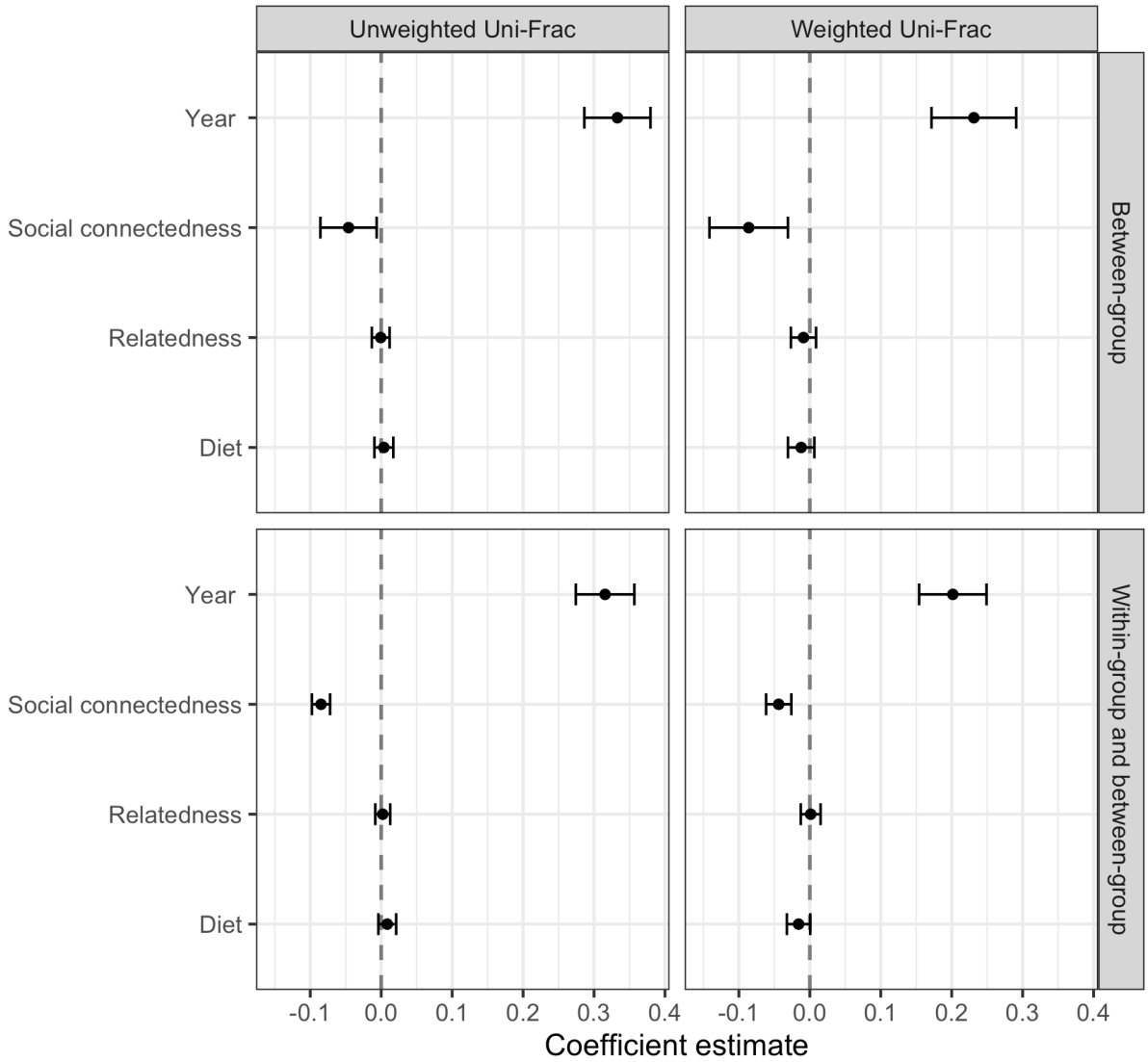
Table 2. The competing GLMMs' fixed effects, Akaike Information Criterion, delta (i.e., difference in AIC between the current model and the best-fit model), and Akaike weights (i.e., relative likelihood of the model), and marginal and conditional R^2 for the best fitting model (i.e., without versus with random effects) when including **a)** within-group and between-group dyads and **b)** only between-group dyads.

To assess whether the effect of social connectedness on gut microbiome beta-diversity was driven by closely connected within-group dyads having very similar gut microbiomes, we repeated the analyses with between-group dyads only (N = 966 dyads). The full models and the social connectedness

319 models were again the strongest supported models (Table 2). Beta-diversity was predicted by year and

320 social connectedness, but not by diet and relatedness (Fig. 2).

321



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323

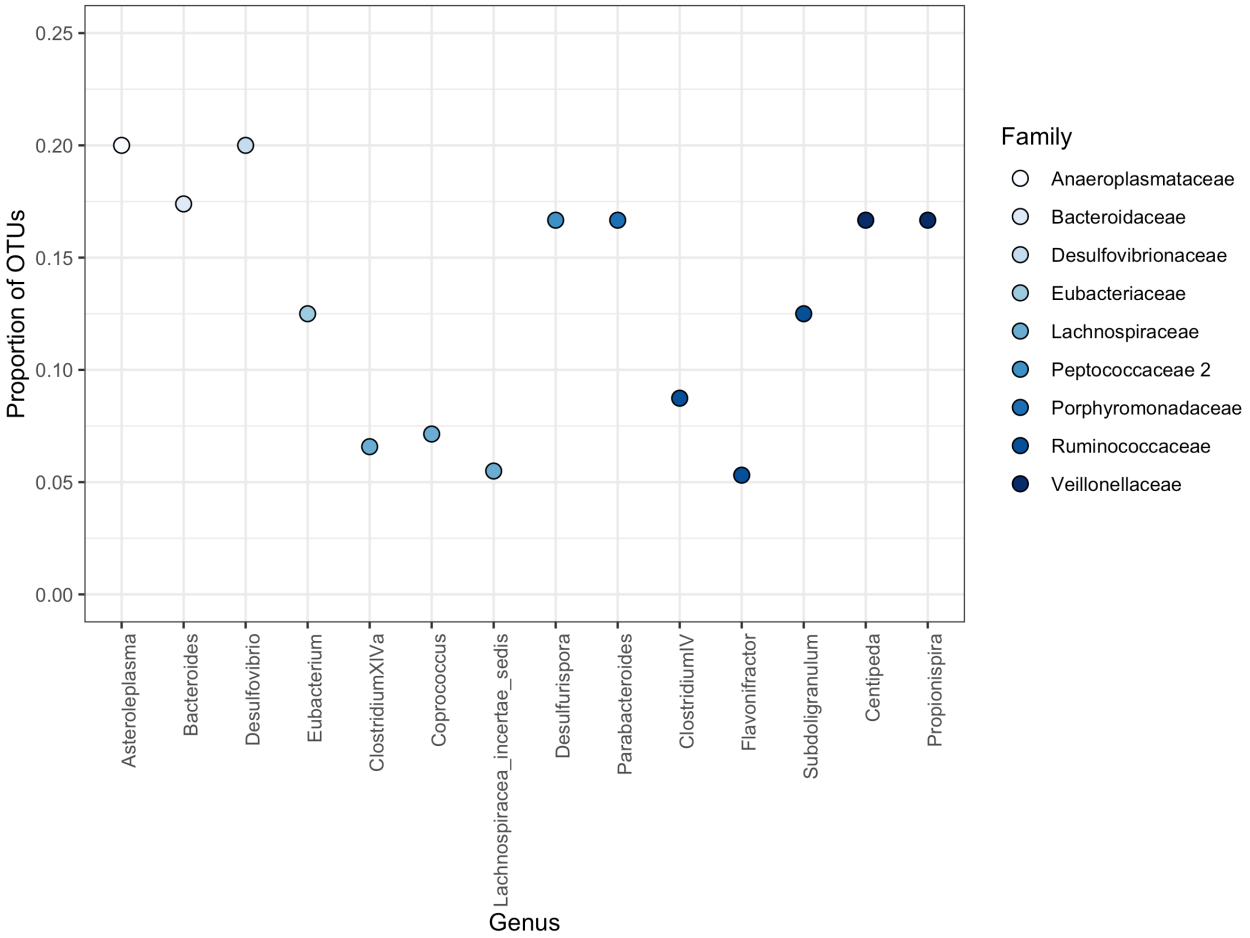
324 **Figure 2.** Coefficient estimates and their 95% confidence intervals for the best fitting model for
325 unweighted versus weighted UniFrac distances in the data set including within-group and between-
326 group dyads and in the data set with between-group dyads only. Social connectedness, dietary
327 dissimilarity, and relatedness ranges from 0 to 1. Of the two study periods, year 2007 was used as the
328 baseline level against which we depict the effect of year 2009.
329
330 *Socially structured OTUs*
331 Our data set contained OTUs from 14 phyla, of which the most well-represented was Firmicutes,
332 followed by Bacteroidetes, Spirochetes, and Verrucomicrobia (Supplementary Material Fig. 1). In each
333 social group, at least 70% of the OTUs belonged to the phylum Firmicutes (Supplementary Material Fig.
334 1) and at least 50% of the OTUs belonged to the families Lachnospiraceae and Ruminococcaceae in the
335 phylum Firmicutes (Supplementary Material Fig. 2).
336 Social connectedness predicted differences in abundances for 73 of the 396 OTUs in the 2009
337 data set (Moran's I range: -0.27 – -0.14, all $P < 0.05$, Supplementary Material Table 1). The number of
338 OTUs with a significant relationship to social connectedness was greater than expected in the phylum
339 Firmicutes (N = 64, Hypergeometric test: $P < 0.001$). The numbers of socially structured OTUs in the
340 phyla Bacteroidetes (N=6), Planctomycetes (N = 1), Proteobacteria (N = 1) and Tenericutes (N = 1) were
341 not greater than expected based on the total number of OTUs in these phyla (Hypergeometric tests, $p >$
342 0.050). The other phyla did not contain any socially structured OTUs. Four families had a higher than
343 expected number of socially structured OTUs: Bacteroidaceae (N = 4), Lachnospiraceae (N = 20),

344 Peptococcaceae 2 (N = 1), and Ruminococcaceae (N = 31). There was also a greater than expected

345 number of socially structured OTUs in 14 of 34 genera (Fig. 4).

346

347



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352 **Figure 4.** The proportion of Operational Taxonomic Units (OTUs) whose abundance was correlated with
353 distance in the proximity network for genera that contained a higher than expected number of socially
354 structured OTUs.
355
356 Social connectedness predicted differences in abundances for 1 of the 450 OTUs in the 2007
357 data set (Moran's I range: -0.27 – -0.14, all $p < 0.05$), which belonged to the phylum Firmicutes, the
358 family Lachnospiraceae, and the genus *Roseburia*. As a result, these taxa had a greater than expected
359 number of socially structured OTUs (Hypergeometric tests, all $p > 0.001$).
360
361 Discussion
362 The aim of this study was to investigate whether the increase in gut microbiome beta-diversity with
363 home range separation in female colobus monkeys was best explained by diet, relatedness, or sociality.
364 Distance in the proximity network was a better predictor than diet and relatedness, similar to findings in
365 more social primates (Amato et al., 2017; Perofsky et al., 2017; Raulo et al., 2017; Tung et al., 2015).
366 Although these previous studies suggest that strong social bonds within social groups drive between-
367 group differences in the gut microbiome after ruling out the effects of relatedness and diet, this is the
368 first report of a relationship between gut microbiome beta-diversity and social connectedness between
369 individuals in different social groups. In contrast, gut microbiome dissimilarity between individuals
370 residing in different social groups did not increase with grooming network distance in sifakas (Perofsky
371 et al., 2017). These contrasting results may be due to the nature or frequency of the host population's

372 between-group interactions. Colobus monkeys sometimes engage in affiliative, sexual, and playful
373 behaviours with non-group members (Supplemental Information; Sicotte & MacIntosh, 2004; Teichroeb,
374 Marteinson, & Sicotte, 2005; Teichroeb et al., 2011), which differ from the almost exclusively aggressive
375 nature of between-group encounters in some other taxa. Similar to these colobus monkeys, mountain
376 gorillas (*Gorilla beringei beringei*) occasionally affiliate with members from other social groups (Forcina
377 et al., 2019) and human foraging societies form extended social networks to optimize resource flow
378 (Hamilton, Milne, Walker, Burger, & Brown, 2007). These extended networks could possibly affect their
379 gut microbiome in similar ways as documented here in colobus monkeys.

380 To determine the consequences of such socially-mediated transmission, the first step is to
381 determine which types of microbes are transmitted this way. The socially transmitted OTUs in this study
382 included all taxa (family Porphyromonadaceae and genera *Parabacteroides* and *Coprococcus)* that
383 diverged after a social group fission at our site (Goodfellow et al., 2019) and genera (*Bacteroides,*
384 *Clostridium,* and *Roseburia*) that were transmitted via grooming and close proximity in howlers (Amato
385 et al., 2017). The close match in socially transmitted taxa in howlers and colobus is not particularly
386 surprising given both have a folivorous diet and low degree of terrestriality, which are factors that
387 influence the gut microbiome (Perofsky et al., 2019). In contrast, the socially transmitted OTUs in our
388 study did not overlap with those transmitted via grooming within social groups of baboons (Tung et al.,
389 2015), despite the host species relatively close phylogenetic relationship. Recent findings show that host
390 phylogeny has a stronger effect than diet on gut microbiome composition (Amato et al., 2019), and it is

391 thus possible that while phylogeny has the strongest overall effect on the gut microbiome, the same gut
392 microbial taxa are structured by sociality in primates with similar lifestyle.

393 We found that the majority of socially transmitted OTUs belonged to the most dominant
394 families in our host population and other folivorous primates (Barelli et al., 2015; Perofsky et al., 2017),
395 the families Lachnospiraceae and Ruminococcaceae in the phylum Firmicutes. These taxa are well-suited
396 for breaking down hard-to-digest plant material (Biddle, Stewart, Blanchard, & Leschine, 2013), and it is
397 therefore possible that socially transmitted gut microbes benefit hosts in terms of improved digestion of
398 mature leaves, which make up the majority of the colobus diet (Saj & Sicotte, 2007). Several studies
399 imply that socially-mediated transmission benefits the host. For example, Tung and colleagues (2015)
400 suggest that the positive health and fitness effects that baboons accrue from forming close social bonds
401 with group members are mediated by the gut microbiome. Our results support the notion that this may
402 be the case in a wide range of gregarious species, including those with relatively low frequencies of
403 social interactions. If social transmission sustains a healthy gut microbiome (as documented in Koch &
404 Schmid-Hempel, 2011), it could provide an incentive for the formation and maintenance of social bonds
405 within social groups (Lombardo, 2008). Our findings leave open the as-of-yet unexplored possibility that
406 social transmission of microbes may even explain the occurrence of friendly between-group encounters,
407 especially in the absence of limiting resources such as fertile females and important food sources.

408 The results of this paper ultimately lead us to an important outstanding question, which is how
409 gut microbes are transmitted among animals that spend considerably less time grooming or in direct
410 contact than other primates with socially mediated gut microbe transmission (Amato et al., 2017; Raulo

411 et al., 2017; Tung et al., 2015). It might be that microbes are transmitted directly during the occasions
412 we observed non-group members copulating, grooming, and playing. However, it could also be that the
413 microbes are transmitted indirectly between hosts when they are touching shared surfaces within a
414 certain time period (Münger et al., 2018). This reasoning is consistent with spatial proximity predicting
415 the gut microbiome in other gregarious species with low frequencies of social behaviours like the Welsh
416 Mountain ponies (*Equus ferus caballus*) (Antwis et al. 2018) and in more solitary species such as North
417 American red squirrels (*Tamiasciurus hudsonicus*) (Ren et al., 2017) and gopher tortoise (*Gopherus*
418 *polyphemus*) (Yuan et al., 2015). The occurrences of direct and indirect social transmission are difficult
419 to tease apart when the two are correlated and when brief physical contact between extra-group
420 members often go unnoticed, but carefully designed studies in the future may be able to address this
421 question.

422 Finally, relatedness and dietary differences within a season were not good predictors of beta-
423 diversity in comparison to social connectedness. In contrast, seasonal changes in diet may be associated
424 with changes in the colobus gut microbiome, because beta-diversity was higher during the 2009 dry
425 season when their diet was more diverse than during the 2007 rainy season when they ate mostly
426 mature leaves. We will continue to investigate whether this seasonal dietary switch is linked to changes
427 in the gut microbiome, as previously reported from other species inhabiting seasonal environments
428 (Amato et al., 2015; Hicks et al., 2018; Orkin, Campos, et al., 2019; Smits et al., 2017; Springer et al.,
429 2017). These authors concluded that gut microbiome dynamics determine nutrient uptake and is key for
430 dietary flexibility (Amato et al., 2015; Hicks et al., 2018; Orkin, Campos, et al., 2019; Smits et al., 2017;

Springer et al., 2017), while the potential three-way interaction between social, dietary, and gut microbial dynamics is still poorly understood. An interesting venue for further research is therefore to investigate whether the gut microbiomes of socially well-connected individuals map more quickly onto ecological changes, which could help them adjust to the rapidly changing environments that many wild animals inhabit today.

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732

733 **Data Accessibility Statement**

734 All raw data are stored in the PaceLab database hosted by the University of Calgary. The 16S sequencing
735 data will be uploaded to NCBI's Short Read Archive. The data used for the analyses presented here will
736 be uploaded to Dryad.

737

738

739 **Appendix**

740 *Microbial taxa predicted to be structured by social connectedness*

741 The last aim of this study was to investigate whether social connectedness was correlated with
742 Operational Taxonomic Unit (OTU) abundances in certain taxa (Table A1) previously reported as
743 structured by social relationships (Amato et al., 2017; Tung et al., 2015). We also expected social
744 connectedness to be correlated with the abundances of three gut microbial taxa that diverged between
745 two daughter groups after a group fission (DA and NP in Fig. A1), because we suspect that this pattern
746 was driven by social network changes (Goodfellow et al., 2019).

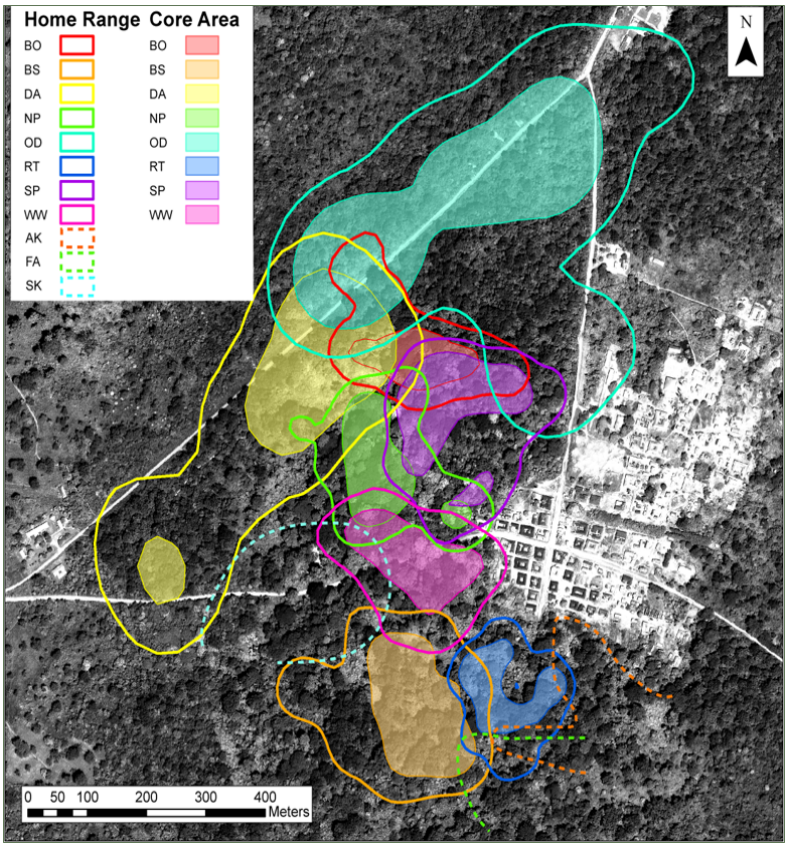


Figure A1. Home ranges and core areas for groups in the main forest fragment at Boabeng-Fiema, Ghana. Solid lines indicate home ranges of groups from which we collected behavioural data. Dashed lines indicate partial home ranges from other groups present in this forest.

752
753

Taxon	Socially structured in 2007	Socially structured in 2009	Reference
Actinobacteria:	X	X	Tung et al. 2015
Bifidobacteriaceae:	NA	X	Tung et al. 2015
Bifidobacterium	NA	X	Tung et al. 2015
Coriobacteriaceae	X	X	Tung et al. 2015
Bacteroidetes:	-	-	-
Bacteroidaceae:	X	(✓)	-
Bacteroides	X	✓	Amato et al. 2017
Porphyromonadaceae:	NA	X	Goodfellow et al. 2019
Parabacteroides	NA	✓	Goodfellow et al. 2019
Firmicutes:	✓	(✓)	-
Clostridiaceae	-	-	-
Clostridium	X	✓	Amato et al. 2017
Eubacteriaceae	-	-	-
Eubacterium	X	(✓)	-
Lachnospiraceae:	✓	(✓)	-
Coprococcus	X	✓	Goodfellow et al. 2019
Lachnospiracea incertae sedis	X	(✓)	-
Roseburia	✓	X	Amato et al. 2017
Peptococcaceae:	X	(✓)	-
Desulfurispora	X	(✓)	-
Ruminococcaceae	X	(✓)	-
Flavonifractor	X	(✓)	-
Subdoligranulum	NA	(✓)	-
Streptococcaceae:	NA	-	-
Streptococcus	NA	NA	Amato et al. 2017
Veillonellaceae:	X	X	Tung et al. 2015
Propionispira	X	(✓)	-
Centipeda	X	(✓)	-
Fuscobacteria	NA	X	Tung et al. 2015
Fusobacteriaceae	NA	X	Tung et al. 2015
Fusobacterium	NA	X	Tung et al. 2015
Proteobacteria:	-	-	-
Desulfovibrionaceae	-	-	-
Desulfovibrio	X	(✓)	-
Enterobacteriaceae	X	X	Tung et al. 2015
Tenericutes:	X	X	Tung et al. 2015
Anaeroplasmataceae	-	-	-
Asteroleplasma	X	(✓)	-
Mycoplasmataceae	NA	X	Tung et al. 2015
Mycoplasma	NA	X	Tung et al. 2015

754 **Table A1.** OTUs in these phyla, families, and genera are expected to be structured by sociality based on

755 previous studies. Predictions were supported ✓; not supported X; no prediction made but structured in

756 our data set (✓); or no prediction made and not structured in our data set (-). Grey text indicates rare
757 taxa (N < 3 OTUs). NA denotes taxa not present in our data set.
758
759 *DNA extraction, amplification, and sequencing protocols for the gut microbiome analysis*
760 We extracted DNA from 200 μl of sample using QIAmp DNA stool extraction protocol the following
761 modifications. Step 2: Added 50 μl Proteinase K with overnight lysis before proceeding to step 3. Step 4:
762 Pipetted all of the supernatant. Step 5: Used half of the InhibitEX tablet. Step 6: Centrifuged for 5
763 minutes. Step 9: Added 4ul RNAse and vortexed for 15 seconds. Step 19: Used 50 μl Buffer AE and
764 incubated at 10 minutes. Step 20: Pipetted the same 50 μl of buffer AE back onto filter and incubated at
765 room temperature for 15 minutes. Centrifuged at full speed for 2 minutes. Our DNA extraction protocol
766 did not include a bead-beating step, which could bias against lysis-resistant taxa such as Gram-positive
767 and spore-forming bacteria that are less likely to be dependent on direct social contact for transmission
768 between hosts because they can survive for prolonged periods outside the host (Pollock, Glendinning,
769 Wisedchanwet, & Watson, 2018; Yuan, Cohen, Ravel, Abdo, & Forney, 2012).
770 We determined the concentration of the extracts using Qubit dsDNA BR Assay Kit (Invitrogen)
771 and diluted products to 2nM for downstream reactions. We amplified the bacterial v4 region of the 16S
772 ribosomal RNA gene using the following 515F and 806R primers containing 5' Illumina adapter tails and
773 dual indexing barcodes:
774 515F 5' AATGATACGGCGACCACCGAGATCTACACTAGATCGCTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
775 806R 5' CAAGCAGAAGACGGCATACGAGATTCACCTAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT.
776 We set the PCRs with 12.5 μl NEB Q5 Hot start 2x Master mix, 1.25 μl 10uM Primer mix, 1 μl template
777 DNA, and 10.25 μl MoBio certified DNA free water and used the following cycling protocol: 98 degrees
778 for 30 seconds (1x) followed by 98 degrees for 10 seconds, 61 degrees for 20 seconds, and 72 degrees
779 for 20 seconds (20x), followed by 72 degrees for 2 minutes and 4 degrees. The amplification products

780 were cleaned up using Ampure XP beads and normalized into a final pool with an Eppendorf liquid
781 handling robot. Libraries were sequenced as part of a 150bp paired-end sequencing run on the Illumina
782 NextSeq platform following the manufacturer's protocol.

783 We used a custom pipeline that contained the following steps: joining pair-end reads; removing
784 low-quality and chimeric reads; dereplication and dropping unique reads with low abundance; clustering
785 OTUs; making OTU table; alignment; building a reference tree; and taxon assignment using FLASH
786 (Magoc & Salzberg, 2011), the FASTX Toolkit (Hannon Lab, 2010), and the USEARCH pipeline (Edgar,
787 2010). See https://github.com/kstagaman/Process_16S and Goodfellow et al. (2019) for further details.
788 We performed *de novo* OTU picking in UCLUST (Edgar, 2010), and sequences with 97% overlap were
789 defined as belonging to the same bacterial Operational Taxonomic Unit (OTU). To guard against
790 sequencing errors, we filtered out OTU's with a frequency lower than 0.00005 as recommended
791 (Bokulich et al., 2012).

792

793 *Variation in predictor and outcome variables*

794 Of the females included in the analyses with behavioural predictor variables (Table A2), dietary
795 dissimilarity (i.e., Sørensen diversity index) varied from 0 to 1, dissimilarity in relatedness calculated as
796 their *R*-value subtracted from 1 ranged from 0.31 to 1, and social connectedness (i.e., inversed path
797 length or Geodesic distance in the 1-meter proximity network) varied from 0 to 1 where 0 represents
798 unconnected dyads (Fig. A2).

799 In our full data set, mean unweighted Unifrac distances within the same season and year was
800 0.052 ± 0.004 for samples collected from the same individual (N = 4 samples) and 0.205 ± 0.042 for
801 samples collected from different individuals within the same season and year (N = 61 samples). The low
802 amount of within-individual variation in comparison to the between-individual variation suggests that
803 one sample per individual is representative of its gut microbiome during that season and sufficient for
804 analysis of beta-diversity. Furthermore, the beta-diversity of matched samples from the same adult

805 female in the wet season 2007 and the dry season 2009 (N = 22 samples from 11 females) was lower

806 (0.152 ± 0.031) than the female's mean beta-diversity with samples from a different female and year

807 (0.183 ± 0.021) for all but one female, and there was a significant difference in beta-diversity between

808 samples from the same versus different females in this sample (N = 11 females, Wilcoxon signed rank

809 test, $p < 0.001$).

810

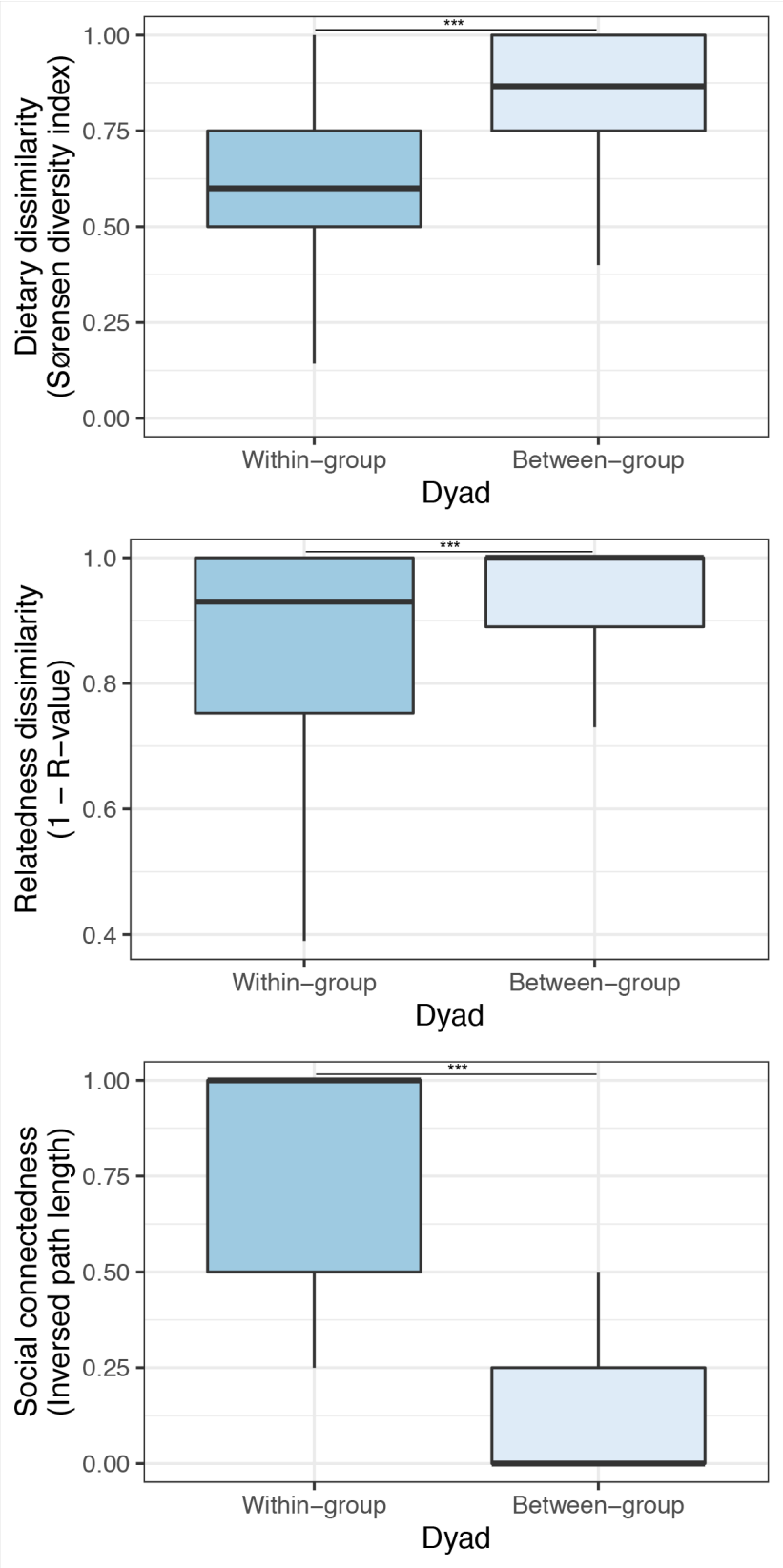
Year	Group	AF group size	AF sampled	AF omitted	Reason for omitting samples
2007	BS	4	4	0	
	DA	5	5	1	Incomplete dietary information
	NP	4	4	0	
	RT	6	5	0	
	SP	4	4	0	
	WW	9	9	0	
2009	BO	8	8	0	
	BS	6	1	1	Lacked samples from majority of AF
	DA	7	2	2	Lacked samples from majority of AF
	NP	5	3	3	Lacked samples from majority of AF
	OD	6	6	0	
	RT	7	2	2	Lacked samples from majority of AF
	SP	3	0	-	
	WW	7	5	0	

811

812 **Table A2.** Number of adult females (AF) present, sampled, and omitted from data analyses with

813 behavioural predictor variables.

814


Dietary dissimilarity
(Sørensen diversity index)
1.00
0.75
0.50
0.25
0.00
***
Within−group
Between−group
Dyad
Relatedness dissimilarity
(1 − R−value)
1.0
0.8
0.6
0.4
***
Within−group
Between−group
Dyad
Social connectedness
(Inversed path length)
1.00
0.75
0.50
0.25
0.00
***
Within−group
Between−group
Dyad

815

816 **Figure A2.** Dietary dissimilarity, relatedness dissimilarity, and social connectedness were lower for

817 within-group than between-group female-female dyads (Wilcoxon signed rank tests, N = 88 samples, all

818 $p < 0.001$).