Social interactions across groups of colobus monkeys (*Colobus vellerosus*) explain similarities in their gut microbiomes

Running title: Social networks predict gut microbiome similarity across groups

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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. We investigated whether diet, relatedness, or 1-meter proximity networks best explains differences in the gut microbiome among 45 female colobus monkeys in 8 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. Group identity explained a large percentage of the variation in gut microbiome beta-diversity. When comparing the predictive power of dietary dissimilarity, relatedness, and distance in the 1-meter proximity network, the models with social distance received the strongest support. Thus, social proximity plays a larger role than diet and relatedness in generating group-level gut microbiome signatures, corroborating previous findings of the importance of social transmission of gut microbes within groups. Our novel findings also show how a social network metric predicts gut microbiome similarity of non-group members, indicating that microbes may be transmitted during intergroup encounters. Lastly, some of the socially transmitted gut microbial taxa are associated with digestion of plant material, but further research is needed to investigate whether social transmission of gut microbes yields health benefits, which could provide an incentive for the formation and maintenance of social bonds within and between 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), and can have important health and fitness consequences for hosts in wild (Koch & Schmid-Hempel, 2011; Vlčková et al., 2018) and captive settings (Turnbaugh et al., 2006). Because of these potential fitness effects, it is important to investigate the acquisition and maintenance of the gut microbiome (Amato, 2016; Archie & Tung, 2015).

The gut microbiome of individuals or groups become more distinct the further apart they live (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 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, we previously reported how the gut microbiomes of colobus monkeys diverged over the course of nine months as a social group fissioned into two daughter groups (Goodfellow et al., 2019). Changes in the kin composition of groups did not explain this pattern, while we were unable to discern whether this divergence was due to the daughter groups

56 having separate home ranges with different food resources or due to the social network changes that
57 resulted from the group fission (Goodfellow et al., 2019). Indeed, few studies have had access to the
58 necessary detailed behavioural, ecological, and genetic data to disentangle whether the divergence in
59 gut microbiomes with home range separation is due to dietary differences, lower degrees of
60 relatedness, or lack of shared social environments (Archie & Tung, 2015; Björk, Dasari, Grieneisen, &
61 Archie, 2019).

62 Diet is suggested to be one of the most important factors affecting the gut microbiome
63 (Voreades, Kozil, & Weir, 2014). The gut microbial composition fluctuates within hosts with seasonal or
64 experimental dietary changes (Davenport et al., 2014; David et al., 2013; Hicks et al., 2018; Leamy et al.,
65 2014; Mallott, Amato, Garber, & Malhi, 2018; Maurice et al., 2015; Michl et al., 2019; Orkin et al., 2019),
66 and dietary similarities may explain whether groups have distinct gut microbiomes (Orkin et al., 2019).
67 However, this pattern could also be due to closer genetic relatedness within than between groups as the
68 host's genetic makeup can affect microbe colonization (Opstal & Bordenstein, 2015; Spor, Koren, & Ley,
69 2011), and a number of genomic regions are associated with gut microbial composition in rodents
70 (Bonder et al., 2016; Leamy et al., 2014). This may explain why closely related individuals have more
71 similar gut microbiomes than unrelated individuals in some studies of humans and captive rodents
72 (Faith et al., 2013; Kovacs et al., 2011; Ley et al., 2005). In contrast, genetic differentiation between
73 populations was a poor predictor of their gut microbiome (Grieneisen et al., 2019), and relatedness did
74 not have a significant effect on the gut microbiome in some studies of humans (Rothschild et al., 2018),
75 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 (Lax et al., 2014). Direct social contact such as grooming or sitting in body contact further increases microbiome transmission between close social partners within 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., 2014). Gut microbiomes are also more similar among socially connected than disconnected siblings and married couples (Dill-McFarland et al., 2019). In contrast, social distance between non-group members did not predict gut microbiome similarity in Verreaux's sifaka (*Propithecus verreauxi*). Taken together, these studies indicate that social interactions transmit gut microbes within groups, while it is unclear whether social interactions between groups have a similar effect.

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). 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). At Boabeng-Fiema, all colobus groups utilize a highly folivorous diet, but the most

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

The frequent between-group interactions coupled with variation in diet and relatedness within and between groups makes this a good study population to investigate whether the pattern of increasing female gut microbial beta-diversity with home range separation is best explained by lower

116 degrees of dietary similarity, relatedness, or social connectedness. We take a cross-sectional approach
117 using observational and genetic data from eight groups to first test whether the gut microbiome was
118 structured by social groups. We predicted gut microbiome beta-diversity to be structured by groups and
119 to increase with home range separation. We then evaluated which factors explained gut microbiome
120 beta-diversity between females across different groups. We expected gut microbiome beta-diversity to
121 be negatively correlated with dietary similarity, relatedness, and connectedness in the 1-meter
122 proximity network. Finally, the significant predictor from the analyses above (social distance) was used
123 in a subsequent population-level analysis of Operational Taxonomic Unit (OTU) abundance to determine
124 which microbial taxa were transmitted this way.
125

126 Materials and Methods

127 *Behavioral data collection*

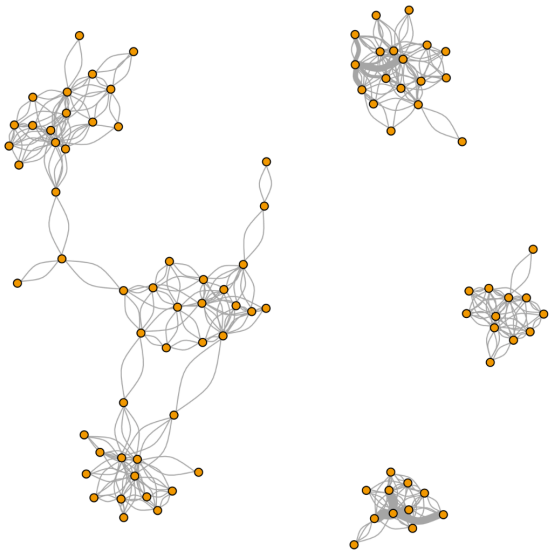
128 Demographic data have been collected since 2000 from the black-and-white colobus monkeys (*Colobus*
129 *vellerosus*) at Boabeng-Fiema, Ghana. In this study, we also use behavioral and ecological data as well as
130 DNA samples from eight groups (Fig. A1) collected during two study periods: the rainy season May-
131 August 2007 and the pre-dry and dry seasons October 2008 - April 2009 (Table A2). During this time
132 period, the study groups contained 4-9 adult (i.e., parous) females (Table A2), 1-4 adult males, and 8-24
133 immatures. Data collection was approved by the Boabeng-Fiema Monkey Sanctuary's management
134 committee, Ghana Wildlife Division, and the University of Calgary's Animal Care Committee (BI 2006-28,
135 BI 2009-25).

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

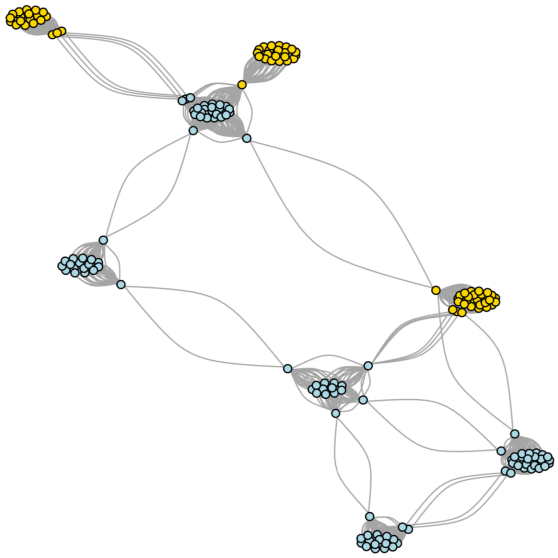
We observed over 300 between-group encounters (i.e., two groups located within 50 meters of each other), of which 53% of the encounters lacked female aggression and 35% of the encounters lacked male aggression. Because close proximity between individuals of different groups are rare and unlikely to be recorded during focal sampling, we recorded approaches to 1 meter collected *ad libitum* (Altmann, 1974). Some of these approaches only led to brief close proximity while others led to prolonged contact like copulations, grooming, and play. We created an undirected proximity network based on the presence and absence of approaches to 1 meter between all individuals (N = 177) present in the eight study groups. We used the software UCINET (Borgatti, Everett, & Freeman, 2002) to compute the dyadic Geodesic distance in the 1-meter proximity network (hereafter referred to as social distance), which is the number of steps (i.e., recorded interaction ties) in the shortest path from one individual to another.

156 Group members were in 1-meter proximity with each other (i.e., a geodesic distance of 1) or separated
157 by two to three partners (i.e., a geodesic distance of 2-4) (Fig. A2). Individuals belonging to different
158 groups had a geodesic distance of 1 to 9 while some were not connected at all and assigned a distance
159 of 10 for the purpose of our data analysis (Fig. 1; Fig. A2). Note that the seemingly unconnected
160 individuals only occurred in the 2007 data set, and they were unconnected because we only had access
161 to data collected from a 3-month period. These individuals are connected and separated with up to
162 eight steps in the 2008-2009 network, which is based on six months of data.
163
164 *Genetic data collection*
165 We collected faecal samples June-August 2007 and January-April 2009. Immedinately after a female
166 defecated, we collected approximatey 1g of feces and dissolved it in 6ml RNAlater. The samples were
167 stored in a fridge at the field site until the end of the field season when they were transported to the
168 Ting lab and stored in a -20-degree C freezer. Note that we lack information on soil type, which was
169 driving between-site differences in the gut microbiomes in a large-scale study of terrestrial baboons
170 (Grieneisen et al., 2019). However, our samples were collected from arboreal primates within a small
171 study area, and sampling site does not have a significant effect on beta-diversity in our study population
172 (Goodfellow et al., 2019).
173

(a) 2007

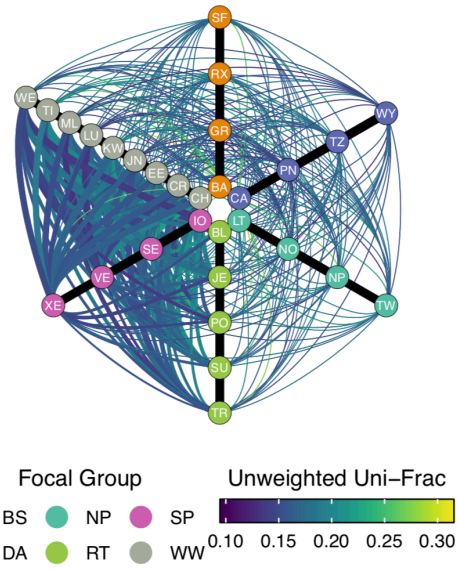


(a) 2009

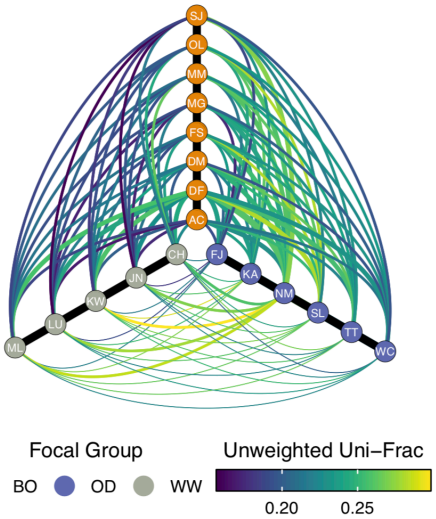


174

(b) 2007



(b) 2009



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176

Figure 1. Social networks for: **a)** the entire population. Each group member is depicted as a node in yellow or blue depending if they resided in a group that was or was not used for behavioural analyses. Between-group dyads with a Geodesic distance of one (i.e., observed in 1-meter proximity) are connected with lines. **b)** females included in the behavioural analyses with lines connecting non-group members (i.e., nodes of different color) where color represents gut microbiome beta-diversity (i.e., unweighted Unifrac distances) ranging from similar (dark) to dissimilar (light) and thickness indicates social distances ranging from short (thick) to long distances (thin).

We extracted DNA from the samples and genotyped the extracts at 17 short tandem repeat loci (STR) as previously described (Wikberg et al., 2012). To make sure that the samples used in the relatedness and gut microbiome analyses were collected from the correct individual, we compared the short tandem repeat STR genotypes obtained from these samples with a second sample collected from the same individual at a different time. We calculated dyadic estimated relatedness values (*R*) in MLRelate (Kalinowski, Wagner, & Taper, 2006) because this method provided the most accurate relatedness estimates in our study population (Wikberg et al., 2012). We used *R*-values calculated from STR loci rather than theoretical relatedness (*r*) calculated from pedigrees, because *R*-values predict kinship relatively accurately in our study population (Wikberg et al., 2014a) and they are more accurate than *r* in studies such as ours with limited access to pedigrees (Forstmeier, Schielzeth, Mueller, Ellegren, & Kempenaers, 2012; Robinson, Simmons, & Kennington, 2013). The median female relatedness was low both within and between groups, but there were at least some closely related females residing in the same 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 used a custom pipeline
205 (https://github.com/kstagaman/Process_16S) for quality filtering and assembly (see Appendix). We
206 performed *de novo* OTU picking in UCLUST (Edgar, 2010), and sequences with 97% overlap were defined
207 as belonging to the same bacterial Operational Taxonomic Unit (OTU). We obtained a total of 2,597
208 OTUs. To guard against sequencing errors, we filtered out OTU's with a frequency lower than 0.00005 as
209 recommended (Bokulich et al., 2012). After filtering, the 2007 data set contained 450 OTUs and had a
210 mean read depth of 91,833 (range: 61,469 – 118,633) and the final 2009 data set contained 396 OTUs
211 with a mean read depth of 91,833 (range: 61,469 – 101,081). We did not rarefy the data set to an even
212 read depth, because it is recommended against (McMurdie & Holmes, 2014).
213 We initially calculated four different measures of gut microbiome beta-diversity (Sørensen
214 dissimilarity index, Bray-Curtis dissimilarity index, unweighted UniFrac distances, and weighted UniFrac
215 distances) in the R package vegan (Oksanen et al., 2017). Because the two presence/absence indices
216 were strongly correlated with each other (Sorenson dissimilarity indices and unweighted UniFrac
217 distances: Mantel $r = 0.93$, $p = 0.001$) as were the two abundance indices (Bray-Curtis dissimilarity

218 indices and weighted UniFrac distances: Mantel r = 0.77, p = 0.001), in our analyses, we only used the
219 one presence/absence index (unweighted UniFrac distances) and the one abundance index (weighted
220 UniFrac distances) that take phylogenetic relationships of OTUs into account.
221
222 *Data analyses*
223 We combined the 2007 and 2009 data sets and included study year (aka season) and individual ID as
224 predictor variables whenever possible (i.e., permutational multivariate analysis of variance and linear
225 mixed models) while we had to create squared interaction matrices for each study year separately when
226 using matrix correlations (i.e., Mantel tests and Moran's test for autospatial correlations). We only used
227 the full data set (N = 61 samples from 45 females) for the initial analysis regarding the effect of group
228 identitiy. All subsequent analyses examined the effects of behavioural variables on beta-diversity in a
229 subset of the full dataset (N = 48 samples from 42 females), as we removed duplicate samples from the
230 same year and female, one adult female with incomplete dietary information, and groups in which the
231 majority of females were not sampled to make sure we had a representative sample of social distance
232 from each group (Table A2).
233 The initial analysis investigated the effects of season, social group, individual identity, and read
234 depth in the full data set (N = 61 samples from 45 females) using permutational multivariate analysis of
235 variance (PERMANOVA) with 10,000 permutations using the adonis function in the R package vegan
236 (Oksanen et al., 2017). The terms were added sequentially in the order listed above.

We used non-parametric Mantel correlations implemented in the R package vegan (Oksanen et al., 2017) to investigate whether the two measures of gut microbiome beta-diversity were correlated with home range separation (0 = same group and home range; 1 = different groups but adjacent home ranges, 2 = different groups and non-adjacent home ranges).

To investigate which combination of dyadic traits predicted gut microbiome beta-diversity between females, we created linear mixed models (LMMs) with the outcome variable gut microbiome beta-diversity and the random effects animal identities. We created a null model that did not contain any fixed effects, alternative models with one fixed effect that represented one of the hypotheses outlined in the introduction (dietary dissimilarities, *R*-values, or social distances), and a full model with all three predictor variables. We included data collection year as a fixed effect in all alternative models because the two sampling years occurred in different seasons and several other studies show strong seasonal shifts in gut microbiome composition (Amato et al., 2015; Hicks et al., 2018; Orkin et al., 2019; Smits et al., 2017; Springer et al., 2017). All numerical predictor variables were centered and scaled (Schielzeth, 2010). We included group and focal identity as random effects in all models. We did not have any issues with collinearity based on low Variance Inflation Factors for the full models (all VIF < 1.43). We evaluated the support for each model using Akaike Information Criterion (AIC) (Akaike, 1974), and this approach allowed us to determine which hypotheses (diet, relatedness, or social distance) was best supported by our data (Burnham & Anderson, 2002). In the first set of analyses, we included dyads that resided in the same group and dyads that resided in different groups. To make sure that the effect of social distance was not driven by the close social bonds within groups, we repeated the analyses with

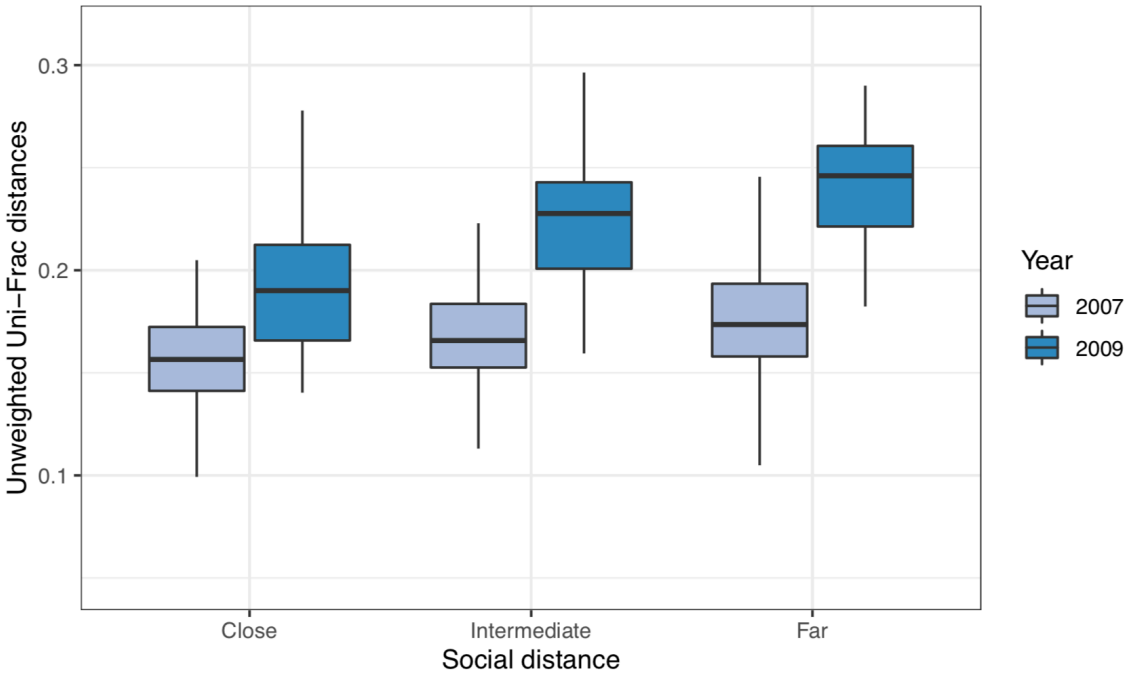
257 between-group dyads only. These analyses were done using the packages lme4 (Bates et al., 2014) and
258 MuMIn (Barton, 2013) in R (R Core Team, 2018).
259 To infer which of the gut microbial taxa may be transmitted via close proximity, which was a
260 better predictor of beta-diversity than diet and relatedness (see Results), we investigated whether the
261 abundance of each OTU was correlated with Geodesic distance in the 1-meter approach network using
262 Moran's test for autospatial correlations implemented in the package ape (Paradis, Claude, & Strimmer,
263 2004). We included within-group and between-group dyads in this analysis (N = 342 dyads). We counted
264 the number of OTUs in each phylum (or family) that were socially structured based on the autospatial
265 correlation results. We conducted hypergeometric tests to investigate whether this number was higher
266 than expected by chance based on the total number of OTUs in the phylum (or family) using the phyper
267 function implemented in R. In all analyses of taxonomic differences, we used the 10% false discovery
268 rate to correct p-values for multiple testing (sensu Tung et al., 2015). The gut microbial taxa we
269 expected to be shaped by sociality are listed in the Table A1 (Amato et al., 2017; Goodfellow et al., 2019;
270 Tung et al., 2015).
271
272 Results
273 *Factors predicting gut microbiome beta-diversity*
274 We investigated the relative effects of season, group, individual ID, and read depth in the full data set
275 (PERMANOVA, N = 61 samples collected 2007-2009). Of the observed variation in the taxonomic
276 composition of the gut microbiome (i.e., beta-diversity), individual identity explained the largest

277 percentage (54-55% depending on which beta-diversity index was used as outcome variable), group
278 identity explained a more moderate percentage (19-28%), while year explained much smaller
279 percentage (8-12%) (Table 1). Read depth did not have a significant effect on beta-diversity (Table 1).
280

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

281 **Table 1.** Results from the PERMANOVA with factors added sequentially in the ordered listed in the table.
282
283 Gut microbiome beta-diversity and home range separation were correlated in the 2007 data set
284 (N = 870 dyads in 6 groups, Mantel tests, unweighted UniFrac distance: r = 0.22, p = 0.002; weighted
285 UniFrac distance: r = 0.10, p = 0.049) and in the 2009 data set (N = 342 dyads in 3 groups, Mantel tests;
286 unweighted UniFrac distance: r = 0.36, p = 0.005; weighted UniFrac distance: r = 0.20, p = 0.024),
287 meaning that females residing farther from each other had less similar gut microbiomes. This pattern
288 can potentially be explained by group members having more similar diets, higher relatedness, or
289 stronger social ties than non-group members (Fig. A2).
290 We created several competing generalized linear mixed models to investigate which of the three
291 hypotheses best explained increasing beta-diversity with home range separation: dietary dissimilarity,

292 relatedness, or social distance, controlling for data collection year. In our data set with both within-
293 group and between-group dyads (N = 1,212 dyads in 2007-2009), the models with social distance
294 received the greatest support (Table 2). Social distance predicted gut microbiome beta-diversity, and
295 females located further apart in the social network had less similar gut microbiomes (Figs. 1-3). Year also
296 predicted gut microbiome beta-diversity (Fig. 3), and females had more similar gut microbiomes during
297 the rainy season of 2007 than the dry season of 2009. Without taking the random effects into account,
298 the model with social distance and year explained 9% to 24% of the observed variation depending on
299 which beta-diversity index was used (Table 2). The alternative models with dietary dissimilarity and
300 relatedness did not receive strong support based on their high AIC values (Table 2).
301



302

303 **Figure 2.** Gut microbiome beta-diversity (i.e., unweighted UniFrac distances) for dyads located close

304 (geodesic distance 1-2), intermediate (geodesic distance 3-4), and far away from each other (geodesic

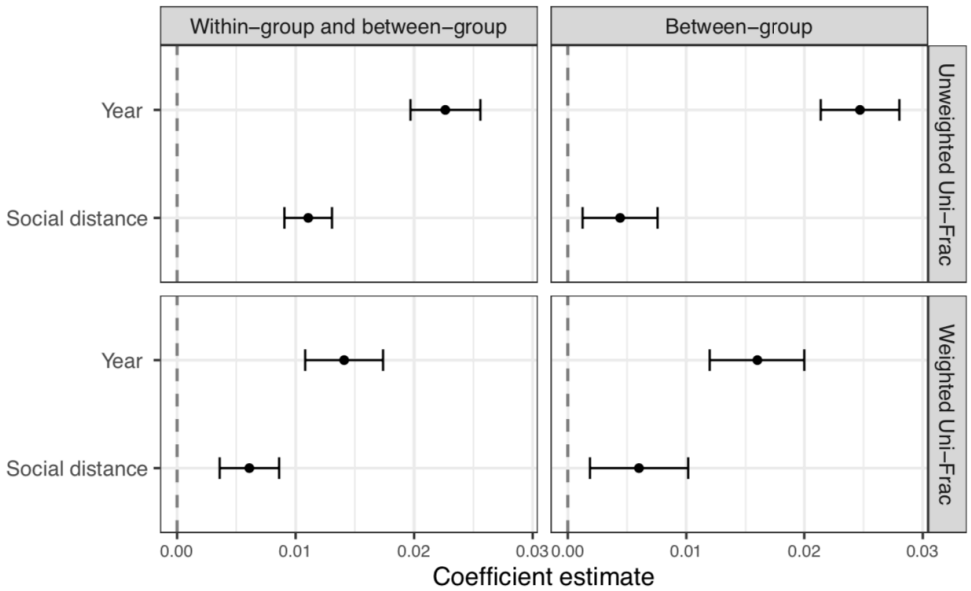
305 distance 5 or greater) in the population's 1-meter proximity network.

306

Outcome variable	Fixed effect	AIC	Delta	Weight	R^2 marginal	R^2 conditional
a) Within-group and between-group dyads						
Unweighted UniFrac	Season + Proximity	-4768.97	0.00	0.84	0.24	0.48
	Season + Diet + Relatedness + Proximity	-4765.69	3.29	0.16		
	Season + Diet	-4714.71	54.26	0.00		
	Season + Relatedness	-4670.38	98.60	0.00		
	-	-4561.86	207.11	0.00		
Weighted UniFrac	Season + Proximity	-4616.00	0.00	1.00	0.09	0.22
	Season + Diet	-4593.90	22.10	0.00		
	Season + Relatedness	-4593.47	22.53	0.00		
	Season + Diet + Relatedness + Proximity	-4588.86	27.13	0.00		
	-	-4572.74	43.26	0.00		
b) Between-group dyads						
Unweighted UniFrac	Season + Proximity	-4230.98	0.00	0.97	0.32	0.56
	Season + Diet	-4223.20	7.79	0.02		
	Season + Relatedness	-4222.57	8.42	0.01		
	Season + Diet + Relatedness + Proximity	-4203.70	27.29	0.00		
	-	-4061.37	169.62	0.00		
Weighted UniFrac	Season + Proximity	-3712.02	0.00	0.94	0.10	0.25
	Season + Diet	-3705.37	6.65	0.03		
	Season + Relatedness	-3704.60	7.42	0.02		
	Season + Diet + Relatedness + Proximity	-3688.41	23.61	0.00		
	-	-3677.10	34.92	0.00		

Table 2. The competing LMMs' 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.

312 To assess whether the effect of social distance on gut microbiome beta-diversity was driven by
313 closely connected within-group dyads having very similar gut microbiomes, we repeated the analyses
314 with between-group dyads only (N = 966 dyads). The social distance models were again the strongest
315 supported models and explained 10% to 32% of the observed variation depending on which beta-
316 diversity index was used as outcome variable (Table 2). Again, both year and social distance predicted
317 beta-diversity (Fig. 3). The alternative models with diet or relatedness were not well supported because
318 of their high AIC values were much higher than those for the proximity models (Table 2).



319

320 **Figure 3.** Coefficient estimates and their 95% confidence intervals for the best fitting model for
321 unweighted versus weighted UniFrac distances in the data set including within-group and between-
322 group dyads and in the data set with between-group dyads only. Social distance is a continuous variable.
323 Of the two study periods, year 2007 was used as the baseline level against which we depict the effect of
324 year 2009.

325

326 *Socially structured OTUs*

327 Our data set contained OTUs from 14 phyla, of which the most well-represented was Firmicutes,
328 followed by Bacteroidetes, Spirochetes, and Verrucomicrobia (Supplementary Material Fig. 1). In each
329 social group, at least 70% of the OTUs belonged to the phylum Firmicutes (Supplementary Material Fig.
330 1) and at least 50% of the OTUs belonged to the families Lachnospiraceae and Ruminococcaceae in the
331 phylum Firmicutes (Supplementary Material Fig. 2).

332 Social distance predicted differences in abundances for 73 of the 396 OTUs in the 2009 data set
333 (Moran's I range: -0.27 – -0.14, all $p < 0.05$, Supplementary Material Table 1). The number of OTUs with
334 a significant relationship to social distance was greater than expected in the phylum Firmicutes (N = 64,
335 Hypergeometric test, $p < 0.001$). The numbers of socially structured OTUs in the phyla Bacteroidetes
336 (N=6), Planctomycetes (N = 1), Proteobacteria (N = 1) and Tenericutes (N = 1) were not greater than
337 expected based on the total number of OTUs in these phyla (Hypergeometric tests, $p > 0.050$). The other
338 phyla did not contain any socially structured OTUs. Four families had a higher than expected number of
339 socially structured OTUs: Bacteroidaceae (N = 4), Lachnospiraceae (N = 20), Peptococcaceae 2 (N = 1),
340 and Ruminococcaceae (N = 31). There was also a greater than expected number of socially structured
341 OTUs in 14 of 34 genera (Fig. 4).

342

343

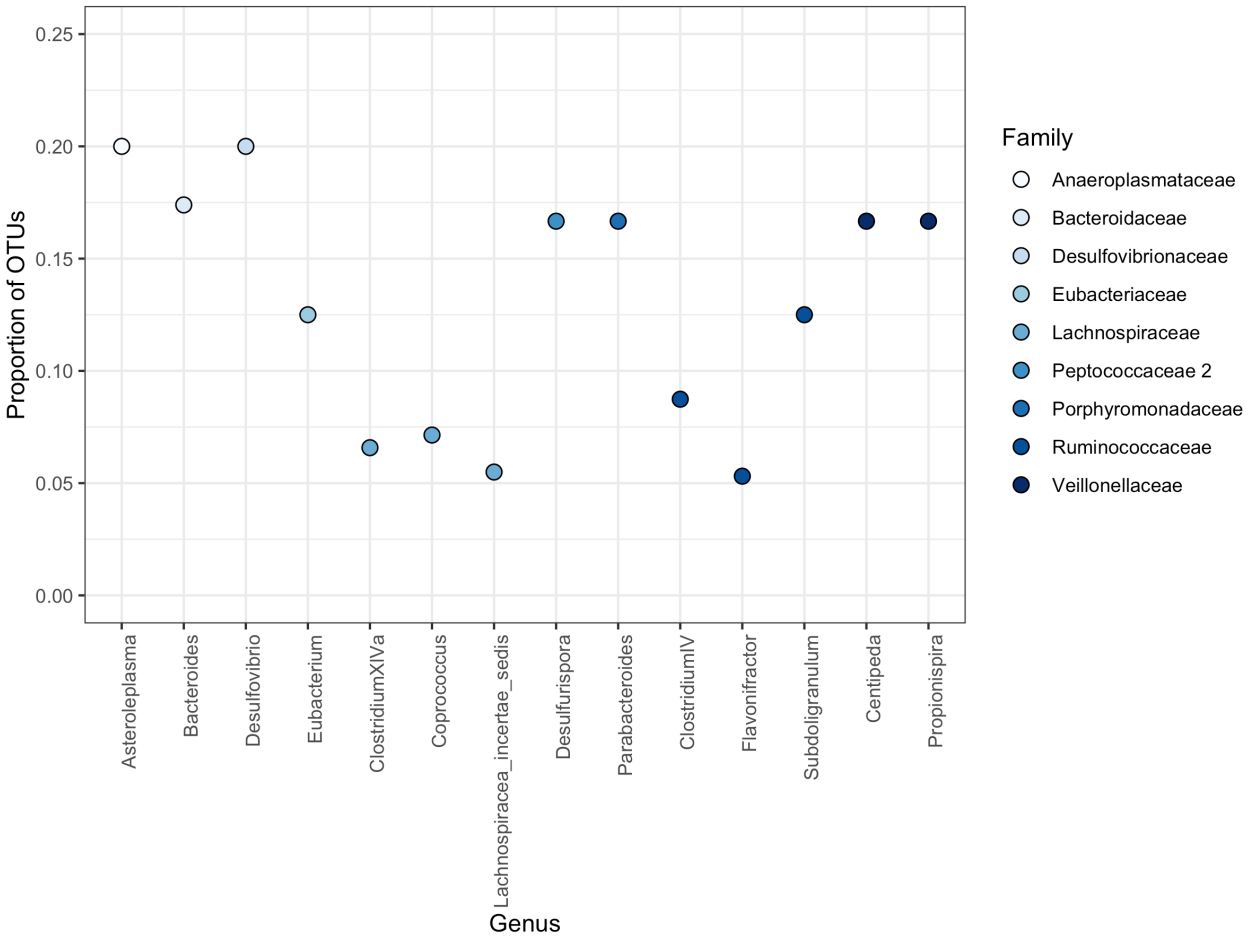


Figure 4. The proportion of Operational Taxonomic Units (OTUs) whose abundance was correlated with distance in the proximity network for genera that contained a higher than expected number of socially structured OTUs.

Social distance predicted differences in abundances for 1 of the 450 OTUs in the 2007 data set (Moran's I range: -0.27 – -0.14, all $p < 0.05$), which belonged to the phylum Firmicutes, the family

351 Lachnospiraceae, and the genus *Roseburia*. As a result, these taxa had a greater than expected number
352 of socially structured OTUs (Hypergeometric tests, all $p > 0.001$).

353

354 Discussion

355 The aim of this study was to investigate if the increase in gut microbiome beta-diversity with home
356 range separation in female colobus monkeys was best explained by diet, relatedness, or sociality.
357 Distance in the proximity network was a better predictor than diet and relatedness, similar to findings in
358 more social primates (Amato et al., 2017; Perofsky, Lewis, Abondano, Di Fiore, & Meyers, 2017; Raulo et
359 al., 2014; Tung et al., 2015). Although these previous studies suggest that strong social bonds within
360 groups drive between-group differences in the gut microbiome after ruling out the effects of relatedness
361 and diet, this is the first report of a relationship between gut microbiome beta-diversity and social
362 connectedness between individuals in different groups. These mostly brief instances of close proximity
363 during between-group encounters explained up to 32% of the variation between non-group members.
364 In contrast, gut microbiome dissimilarity between individuals residing in different groups did not
365 increase with grooming network distance in sifakas (Perofsky et al., 2017). These contrasting results may
366 be due to the nature of the host population's between-group interactions, particularly the presence or
367 absence of friendly behaviours. Colobus monkeys sometimes engage in affiliative, sexual, and playful
368 behaviours with non-group members (Supplemental Information; Sicotte & MacIntosh, 2004; Teichroeb,
369 Marteinson, & Sicotte, 2005; Teichroeb et al., 2011), which differ from the almost exclusively aggressive
370 nature of between-group encounters in some other taxa. Similar to these colobus monkeys, mountain

371 gorillas (*Gorilla beringei beringei*) occasionally affiliate with members from other groups (Forcina et al.,
372 2019) and human foraging societies form extended social networks to optimize resource flow (Hamilton,
373 Milne, Walker, Burger, & Brown, 2007). These extended networks could possibly affect their gut
374 microbiome in similar ways as documented here in colobus monkeys.

375 To determine the consequences of such social transmission, the first step is to determine which
376 type of microbes are transmitted this way. The socially transmitted OTUs in this study included all taxa
377 (family Porphyromonadaceae and genera *Parabacteroides* and *Coprococcus)* that diverged after a group
378 fission at our site (Goodfellow et al., 2019) and genera (*Bacteroides, Clostridium,* and *Roseburia*) that
379 were transmitted via grooming and close proximity in howlers (Amato et al., 2017). In contrast, the
380 socially transmitted OTUs in our study did not overlap with those transmitted via grooming within
381 groups of baboons (Tung et al., 2015), despite the host species relatively close phylogenetic relationship.
382 This discrepancy with the baboon study is not particularly surprising given that the colobus lifestyle is
383 more similar to howlers than baboons, including factors that influence the gut microbiome like diet and
384 degree of terrestriality (Perofsky, Lewis, & Meyers, 2019). This is despite recent findings that host
385 phylogeny has a stronger effect than diet on gut microbiome composition (Amato et al., 2019). It is thus
386 possible that while phylogeny has the strongest overall effect on the gut microbiome, the same gut
387 microbial taxa are structured by sociality in primates with similar lifestyle.

388 We found that the majority of socially transmitted OTUs belonged to the most dominant
389 families in our host population and other folivorous primates (Barelli et al., 2015; Perofsky et al., 2017),
390 the families Lachnospiraceae and Ruminococcaceae in the phylum Firmicutes. These taxa are well-suited

for breaking down hard-to-digest plant material (Biddle, Stewart, Blanchard, & Leschine, 2013), and it is therefore possible that socially transmitted gut microbes benefit hosts in terms of improved digestion of mature leaves, which make up the majority of the colobus diet (Saj & Sicotte, 2007). Several studies imply that social gut microbe transmission benefits the host. For example, Tung and colleagues (2015) suggest that the positive health and fitness effects that baboons accrue from forming close social bonds with group members are mediated by the gut microbiome. Our results support the notion that this may be the case in a wide range of gregarious species, including those with relatively low frequencies of social interactions. If social transmission sustains a healthy gut microbiome (as documented in Koch & Schmid-Hempel, 2011; Vlčková et al., 2018), it could provide an incentive for the formation and maintenance of social bonds within groups (Lombardo, 2008). Our findings leave open the as-of-yet unexplored possibility that social transmission of microbes may even explain the occurrence of friendly between-group encounters, especially in the absence of limiting resouces such as fertile females and important food sources.

The results of this paper ultimately lead us to an important outstanding question, which is how gut microbes are transmitted among animals that spend considerably less time grooming or in direct contact than other primates with socially mediated gut microbe transmission (Amato et al., 2017; Raulo et al., 2014; Tung et al., 2015). It might be that microbes are transmitted directly during the occasions we observed non-group members copulating, grooming, and playing. However, it could also be that the microbes are transmitted indirectly between hosts when they are touching shared surfaces within a certain time period (Münger, Montiel-Castro, Langhans, & Pacheco-López, 2018). This reasoning is

411 consistent with spatial proximity predicting the gut microbiome in other gregarious species with low
412 frequencies of social behaviours like the Welsh Mountain ponies (*Equus ferus caballus*) (Antwis, Lea,
413 Unwin, & Shultz, 2018) and in more solitary species such as North American red squirrels (*Tamiasciurus*
414 *hudsonicus*) (Ren et al., 2017) and gopher tortoise (*Gopherus polyphemus*) (M. L. Yuan et al., 2015). The
415 occurrences of direct and indirect social transmission are difficult to tease apart when the two are
416 correlated and when brief physical contact between extra-group members often go unnoticed, but
417 carefully designed studies in the future may be able to address this question.

418 Finally, relatedness and dietary differences within a season were not good predictors of beta-
419 diversity in comparison to social distance. In contrast, seasonal changes in diet may be associated with
420 changes in the colobus gut microbiome, because beta-diversity was higher during the 2009 dry season
421 when their diet was more diverse than during the 2007 rainy season when they ate mostly mature
422 leaves. We will continue to investigate whether this seasonal dietary switch is linked to changes in the
423 gut microbiome, as previously reported from other species inhabiting seasonal environments (Amato et
424 al., 2015; Hicks et al., 2018; Orkin et al., 2019; Smits et al., 2017; Springer et al., 2017). These authors
425 concluded that gut microbiome dynamics determine nutrient uptake and is key for dietary flexibility
426 (Amato et al., 2015; Hicks et al., 2018; Orkin et al., 2019; Smits et al., 2017; Springer et al., 2017), while
427 the potential three-way interaction between social, dietary, and gut microbial dynamics is still poorly
428 understood. An interesting venue for further research is therefore to investigate whether the gut
429 microbiomes of socially well-connected individuals map more quickly onto ecological changes, which
430 could help them deal with the rapidly changing environments that many wild animals inhabit today.

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717 and Purification of DNA from the Human Microbiome. *PLOS ONE*, *7*(3), e33865. doi:
718 10.1371/journal.pone.0033865

719

720 **Data Accessibility Statement**

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

724

725 **Author contribution**

726 E.C.W., P.S., and N.T. designed and funded the study. E.C.W. and D.C. conducted the DNA extractions
727 and analysed the data. All authors contributed to writing the manuscript.

728

729 **Appendix**

730 *Microbial taxa predicted to be structured by social distance*

731 The last aim of this study was to investigate whether social distance was correlated with Operational
732 Taxonomic Unit (OTU) abundances in certain taxa (Table A1) previously reported as structured by social
733 relationships (Amato et al., 2017; Tung et al., 2015). We also expected social distance to be correlated
734 with the abundances of three gut microbial taxa that diverged between the two daughter groups after a

group fission (DA and NP in Fig. A1), because we suspect that this pattern was driven by social network changes (Goodfellow et al., 2019).

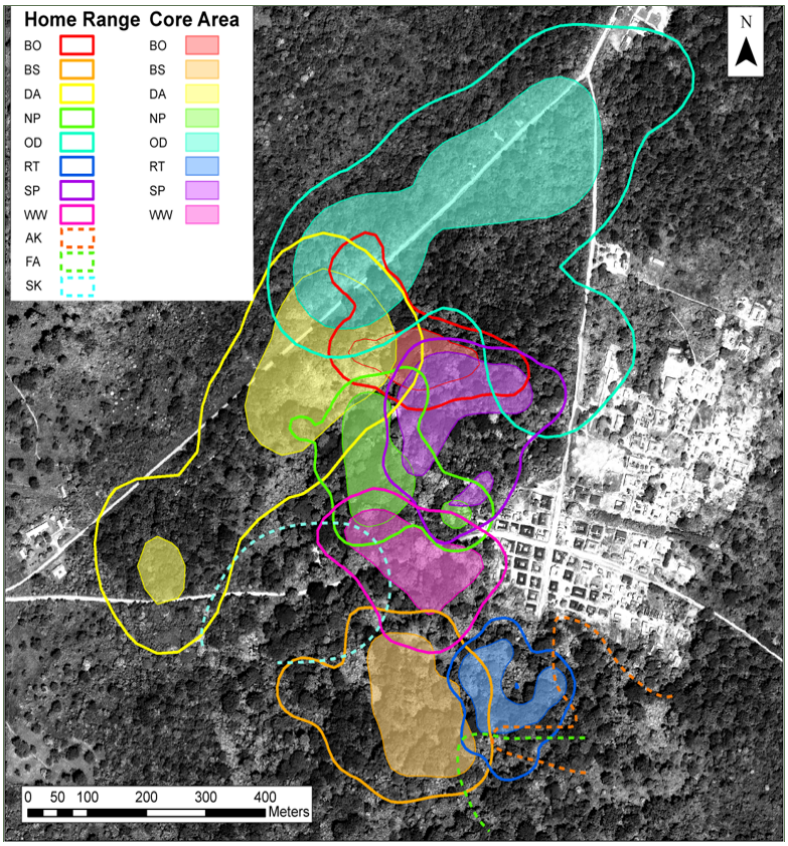

Figure A1. Home ranges and core areas for groups in the main forest fragment at Boabeng-Fiema, Ghana. Solid lines indicate home ranges of groups from which we collected behavioural data. Dashed lines indicate partial home ranges from other groups present in this fores

742
743

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

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

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

746 our data set (✓); or no prediction made and not structured in our data set (-). Grey text indicates rare
747 taxa (N < 3 OTUs). NA denotes taxa not present in our data set.
748
749 *DNA extraction, amplification, and sequencing protocols for the gut microbiome analysis*
750 We extracted DNA from 200 μl of sample using QIAmp DNA stool extraction protocol the following
751 modifications. Step 2: Added 50 μl Proteinase K. Step 4: Pipetted all of the supernatant. Step 5: Used
752 half of the InhibitEX tablet. Step 6: Centrifuged for 5 minutes. Step 9: Added 4ul RNAse and vortexed for
753 15 seconds. Step 19: Used 50 μl Buffer AE and incubated at 10 minutes. Step 20: Pipetted the same 50 μl
754 of buffer AE back onto filter and incubated at room temperature for 15 minutes. Centrifuged at full
755 speed for 2 minutes. Our DNA extraction protocol did not include a bead-beating step, which could bias
756 against lysis-resistant taxa such as Gram-positive and spore-forming bacteria that are less likely to be
757 dependent on direct social contact for transmission between hosts because they can survive for
758 prolonged periods outside the host (Pollock, Glendinning, Wisedchanwet, & Watson, 2018; Yuan, Cohen,
759 Ravel, Abdo, & Forney, 2012).
760 We determined the concentration of the extracts using Qubit dsDNA BR Assay Kit (Invitrogen)
761 and diluted products to 2nM for downstream reactions. We amplified the bacterial v4 region of the 16S
762 ribosomal RNA gene using the following 515F and 806R primers containing 5' Illumina adapter tails and
763 dual indexing barcodes:
764 515F 5' AATGATACGGCGACCACCGAGATCTACACTAGATCGCTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
765 806R 5' CAAGCAGAAGACGGCATACGAGATTCACCTAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT.
766 We set the PCRs with 12.5 μl NEB Q5 Hot start 2x Master mix, 1.25 μl 10uM Primer mix, 1 μl template
767 DNA, and 10.25 μl MoBio certified DNA free water and used the following cycling protocol: 98 degrees
768 for 30 seconds (1x) followed by 98 degrees for 10 seconds, 61 degrees for 20 seconds, and 72 degrees
769 for 20 seconds (20x), followed by 72 degrees for 2 minutes and 4 degrees. The amplification products

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

773 We used a custom pipeline that contained the following steps: joining pair-end reads; removing
774 low-quality and chimeric reads; dereplication and dropping unique reads with low abundance; clustering
775 OTUs; making OTU table; alignment; building a reference tree; and taxon assignment using FLASH
776 (Magoc & Salzberg, 2011), the FASTX Toolkit (Hannon Lab, 2010), and the USEARCH pipeline (Edgar,
777 2010). See https://github.com/kstagaman/Process_16S and Goodfellow et al. (2019) for further details.
778 We performed *de novo* OTU picking in UCLUST (Edgar, 2010), and sequences with 97% overlap were
779 defined as belonging to the same bacterial Operational Taxonomic Unit (OTU). After these steps, we had
780 a total of 2,597 OTUs and a mean read depth of 91,833 (range: 68,252 – 120,119). To guard against
781 sequencing errors, we filtered out OTU's with a frequency lower than 0.00005 as recommended
782 (Bokulich et al., 2012).

783

784 *Variation in predictor and outcome variables*

785 Of the females included in the analyses with behavioural predictor variables (Table A2), dietary
786 dissimilarity (i.e., Sørensen diversity index) varied from 0 to 1, dissimilarity in relatedness calculated as
787 their *R*-value subtracted from 1 ranged from 0.31 to 1, and social distance (i.e., Geodesic distance in the
788 1-meter proximity network) varied from 1 to 10 where 10 represents unconnected dyads (Fig. A2).

789 In our full data set, mean unweighted Unifrac distances within the same season and year was
790 0.052 ± 0.004 for samples collected from the same individual (N = 4 samples) and 0.205 ± 0.042 for
791 samples collected from different individuals within the same season and year (N = 82 samples). The low
792 amount of within-individual variation in comparison to the between-individual variation suggests that
793 one sample per individual is representative of its gut microbiome during that season and sufficient for
794 analysis of beta-diversity. Furthermore, the beta-diversity of matched samples from the same adult

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

796 (0.152 ± 0.031) than the female's mean beta-diversity with samples from a different female and year

797 (0.183 ± 0.021) for all but one female, and there was a significant difference in beta-diversity between

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

799 test, $p < 0.001$).

800

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

801

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

803 behavioural predictor variables (i.e., Mantel tests and LMMs).

804

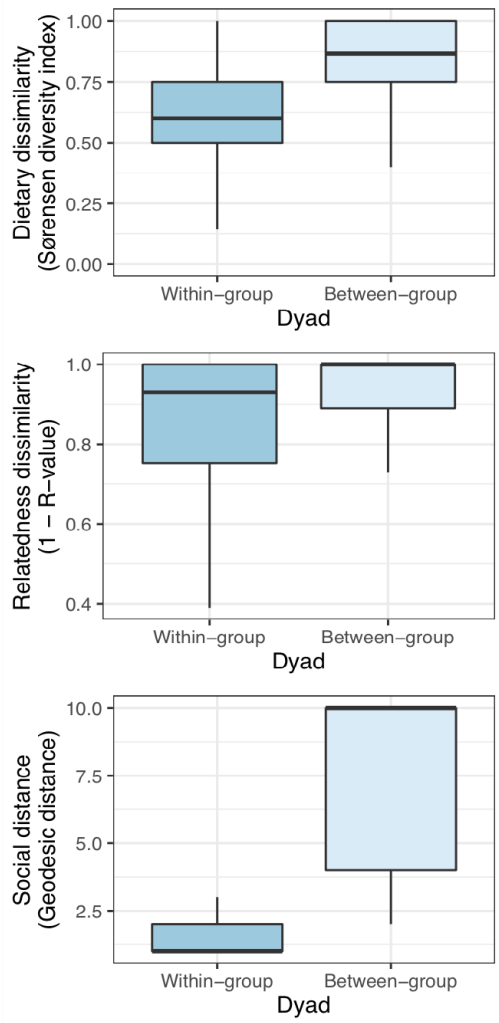

Figure A2. Variation in dietary dissimilarity, relatedness dissimilarity, and social distance for within-group and between-group dyads.