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RESEARCH ARTICLE

Household composition and the infant fecal microbiome: The INSPIRE study

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Abstract

Objectives: Establishment and development of the infant gastrointestinal microbiome (GIM) varies cross-culturally and is thought to be influenced by factors such as gestational age, birth mode, diet, and antibiotic exposure. However, there is little data as to how the composition of infants' households may play a role, particularly from a cross-cultural perspective. Here, we examined relationships between infant fecal microbiome (IFM) diversity/composition and infants' household size, number of siblings, and number of other household members.

Materials and methods: We analyzed 377 fecal samples from healthy, breastfeeding infants across 11 sites in eight different countries (Ethiopia, The Gambia, Ghana, Kenya, Peru, Spain, Sweden, and the United States). Fecal microbial community structure was determined by amplifying, sequencing, and classifying (to the genus level) the V1–V3 region of the bacterial 16S rRNA gene. Surveys administered to infants' mothers identified household members and composition.

Results: Our results indicated that household composition (represented by the number of cohabitating siblings and other household members) did not have a measurable impact on the bacterial diversity, evenness, or richness of the IFM. However, we observed that variation in household composition categories did correspond to differential relative abundances of specific taxa, namely: *Lactobacillus*, *Clostridium*, *Enterobacter*, and *Klebsiella*.

Discussion: This study, to our knowledge, is the largest cross-cultural study to date examining the association between household composition and the IFM. Our results indicate that the social environment of infants (represented here by the proxy of household composition) may influence the bacterial composition of the infant GIM, although the mechanism is unknown. A higher number and diversity of cohabitants and potential caregivers may facilitate social transmission of beneficial bacteria to the infant gastrointestinal tract, by way of shared environment or through direct

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physical and social contact between the maternal–infant dyad and other household members. These findings contribute to the discussion concerning ways by which infants are influenced by their social environments and add further dimensionality to the ongoing exploration of social transmission of gut microbiota and the "old friends" hypothesis.

KEYWORDS
gastrointestinal, horizontal transmission, microbiota, siblings, social transmission

1 | INTRODUCTION

Microbiome research increasingly emphasizes the dual roles of both evolutionary and environmental factors in shaping the human gastrointestinal microbiome (GIM; Davenport et al., 2017). Although foundations of the GIM are likely evolutionarily conserved, dietary and environmental factors continuously modify it throughout an individual's lifespan, and it is in early development (<1-year postpartum) that the GIM is the most susceptible to compositional changes (Arrieta, Stiemsma, Amenyogbe, Brown, & Finlay, 2014). Differential patterns of establishment and succession during this critical period are thought to lay the long-standing foundations of the more stable adult GIM, which is typically characterized by higher bacterial richness and diversity (Yatsunenko et al., 2012). Studies conducted in primarily Western populations indicate the infant GIM (<1-year postpartum) is dominated by several core genera: *Bifidobacterium*, *Lactobacillus*, *Streptococcus*, *Faecalibacterium*, *Lachnospiraceae*, *Blautia*, *Clostridium*, *Veillonella*, *Escherichia/Shigella* (indistinguishable using 16S methodology), *Pasteurellales*, and *Bacteroides* (Milani et al., 2017). Yet, an increasing number of studies conducted in non-Western populations indicate that geographic location is a strong predictor of variation in GIM profiles (Kaul, Davidov, & Peddada, 2017; Lackey et al., 2019; Yatsunenko et al., 2012).

1.1 | Establishment of the infant GIM

Vertical and horizontal modes of transmission lay the initial foundation of the developing infant GIM, potentially as early as in utero via placental transfer (Aagaard et al., 2014); however, there is still debate regarding the existence of viable bacteria in the placenta (Lauder et al., 2016). Following potential in utero transfer, gestational age (premature vs. full-term; Hill et al., 2017), delivery mode (vaginal vs. cesarean; Dominguez-Bello et al., 2010), and feeding pattern (exclusively breastfed, formula, or mixed feeding; Martin et al., 2016; O'Sullivan, Farver, & Smilowitz, 2015) are the primary perinatal drivers of the infant GIM. Variation in these factors during critical periods of early development may lead to differential patterns of bacterial colonization and diversity, potentially influencing long-term health outcomes (Bager, Wohlfahrt, & Westergaard, 2008; Jakobsson et al., 2014; Mueller et al., 2015).

Additionally, maternal body mass index (BMI) and diet during pregnancy may influence patterns of establishment in the GIM (Chu et al., 2016; Collado, Isolauri, Laitinen, & Salminen, 2010); however, the observed influence of maternal BMI and diet on the infant GIM may simply reflect confounding associations among maternal BMI, parity, and breastfeeding patterns (Milani et al., 2017).

1.2 | Environmental influences on the infant GIM

In addition to vertical modes of transmission, the infant GIM may be continuously modified by environmental exposures; for example, maternal and/or infant antibiotic exposure is associated with a reduction in overall taxonomic richness and diversity of the infant GIM (Nogacka et al., 2017; Yasmin et al., 2017). Additionally, GIM composition may be influenced by pet ownership and proximity to animals (Azad et al., 2013; Tun et al., 2017), parental sanitation practices (pacifier cleaning method: Hesselmar et al., 2013; use of specific household products: Ribado et al., 2017) and other environmental factors varying by geographic location (Grześkowiak et al., 2012; Lin et al., 2013; Stearns et al., 2017).

1.3 | Social transmission of microbiota

Social factors such as family size and number of siblings are also considered potential influences on the infant GIM, typically in the context

of predicting childhood atopic outcomes (Adlerberth et al., 2007; Azad et al., 2013; Laursen et al., 2015; Penders et al., 2013). The notion that larger family size might affect the infant GIM is partially inspired by Rook's "old friends" hypothesis (Rook et al., 2004; Rook & Brunet, 2005), a revised version of Strachan's "hygiene hypothesis" (1989). Strachan (1989), noting the inverse relationship between household size and prevalence of hay fever and eczema, suggested that infection in early development (obtained through physical contact with older siblings) subsequently leads to reduced incidence of allergies, an association later replicated (Karmaus & Botezan, 2002; Strachan et al., 2015). In contrast, Rook (2010) suggests that as opposed to infection, it is merely exposure to diverse microbes during critical periods of development that facilitate immune recognition, and thus reduce subsequent development of allergy. Although the underlying mechanisms proposed by Strachan and Rook differ, both frameworks point to evidence suggesting that larger family sizes and sibling presence appear to confer benefits to infant immunity, perhaps mediated in part by modulation of the infant microbiome. However, there are significant gaps in our understanding of how the social worlds of infants differentially facilitate the horizontal transmission of microbiota.

In the nonhuman animal world, there are several examples indicating socially mediated transfer of microbiota, sometimes with a demonstrated adaptive benefit to the host. For example, individual fecal microbiome composition is strongly predicted by social group membership in a variety of animals, including bees (Koch & Schmid-Hempel, 2011), ants (Anderson et al., 2012), lemurs (Raulo et al., 2017), baboons (Tung et al., 2015), and chimpanzees (Degnan et al., 2012; Moeller et al., 2016). In some cases, frequency of physical social interaction or time spent in social contact is associated with the level of interindividual GIM similarity (controlling for kinship, diet, and shared environment), in support of a socially mediated mechanism of transmission (Amato et al., 2017; Moeller et al., 2016; Tung et al., 2015). In a social bee species, a direct adaptive benefit to the social transmission of mutualistic gut bacteria has been observed; without the presence of a specific GI bacterium, the host bee is left susceptible to a highly virulent gut parasite (Koch & Schmid-Hempel, 2011).

There is also evidence of socially mediated bacteria in the human world. Individuals belonging to the same household harbor more similar microbial communities than those in different households (fecal: Claesson et al., 2012; Song et al., 2013; skin: Lax et al., 2014), although it remains unclear whether this overlap was due to direct horizontal transmission or simply shared environment. However, physical social contact has been shown to directly facilitate interindividual similarity with respect to oral (Kort et al., 2014) and skin (Meadow, Bateman, Herkert, O'Connor, & Green, 2013) microbial communities. Thompson, Monteagudo-Mera, Cadenas, Lampl, and Azcarate-Peril (2015) observed that the GIM of daycare attending infants (who likely come into physical contact with a more diverse group of individuals at a higher frequency than infants raised solely at home) was more diverse than that of the non-daycare attending infants in their sample. Additionally, Schnorr et al. (2014) found higher intragroup similarity of adult fecal microbiome composition in a Hadza forager population than in an urban Italian population, which may correspond to patterns of denser cohabitation and food sharing.

1.4 | Household composition and the infant GIM

Household composition can be considered an indicator of how many and which individuals are likely physically interacting with, caring for, and sharing a common environment with infants on a regular basis, and therefore is often used as a predictor of socially mediated microbiota transmission, yet findings have been inconsistent. The number of siblings in the home has been found to be positively associated with GIM richness and diversity (Laursen et al., 2015); however, it has also been observed that older sibling presence negatively correlates with GIM richness and diversity (Azad et al., 2013). Family size (measured by child presence) has been observed to have no relationship with household member fecal microbiome diversity (Song et al., 2013); but in another study, number of family members was positively associated with relative abundance of *Bifidobacterium* in the IFM (Sjögren, Jenmalm, Böttcher, Björkstén, & Sverremark-Ekström, 2009). The number of siblings has been observed to be positively associated with increased *Lactobacillus* (Penders et al., 2013), *Bifidobacterium* (Hasegawa et al., 2017; Penders et al., 2006; Yap et al., 2011), and *Bacteroides* (Laursen et al., 2015; Penders et al., 2013) and lower clostridia colonization of the IFM (Penders et al., 2013). Lack of siblings has also been found to be associated with earlier colonization of *Clostridium* species (Adlerberth et al., 2007; Penders et al., 2006). Inconsistencies in the human studies may be explained by differences in fecal sample collection and microbial sequencing methodologies, and heterogeneity with respect to controlling for potentially confounding variables (e.g., birth mode, breastfeeding practices, and environmental exposures).

Here, we present an exploratory examination of the relationship between household composition and the IFM, the most reliable and available representation of the infant lower GIM (Gill et al., 2006), with respect to both bacterial diversity, evenness, and richness levels (alpha diversity) and overall taxonomic composition. Consistent with the expectations of a socially mediated microbiota transmission mechanism, we hypothesized that the number of siblings, number of extended family members, and household size would be positively associated with increased IFM diversity, evenness, and richness. We also hypothesized that due to household members likely differing in their capacity to be a vector of socially mediated bacteria, household size, sibling presence/number, and extended family presence/number are associated with variation in the relative abundances of bacteria in the IFM.

2 | MATERIALS AND METHODS

2.1 | Study populations and design

Data used in these analyses were collected as part of a larger, cross-sectional study, which broadly sought to characterize the IFM in 11 populations, including two from the United States (ethnically heterogeneous residents of Southeastern Washington and Northwestern Idaho and self-identified Hispanic residents of Southern California),

one South American (Peruvian, residing in a peri-urban area of Lima), two European groups (Swedish, recruited near Helsingborg and self-identified as having Nordic heritage; and Spanish, recruited in Madrid, Zaragoza, Huesca, and Vizcaya with no conditions regarding ethnicity), and six sub-Saharan African groups (rural and urban Ethiopian, rural and urban Gambian, Kenyan, and Ghanaian). The rural and urban Ethiopian participants were recruited from the highlands and city of Hawassa, respectively, both locations within the Southern Nations, Nationalities, and Peoples' Region. Both rural and urban Gambian participants self-identified as Mandinka and were recruited from the West Kiang and Bakau regions, respectively. Kenyan participants were enrolled in the ethnically diverse city of Nakuru. Ghanaian participants resided in southeastern Ghana, and self-identified as either Krobo or Dangme. These populations have been previously described elsewhere (McGuire et al., 2017; Ruiz et al., 2017).

Infants enrolled in the study were between the ages of 2 weeks and 5 months, described by their mothers to be healthy, showing no symptoms of illness in the week prior to enrollment, and having not received antibiotics in the 30 days prior to enrollment. Mothers were also required to be healthy, showing no symptoms of acute illness in the week prior to enrollment, not experiencing breast infection or atypical breast pain, and having not received antibiotics in the month prior to enrollment. Inclusion in the study did not require that infants be exclusively breastfed, but maternal–infant dyads were required to be breastfeeding and/or pumping at least five times per day. Ethics approvals were obtained from each of the participating institutions, as well as overarching approval from the Washington State University Institutional Review Board (#13264). Samples and data were collected between May 2014 and April 2016.

2.2 | Infant fecal sample collection

Infant fecal samples were collected by study personnel during the collection visit or, if sample collection was not possible during the visit, samples were subsequently collected by the infants' mothers according to the standardized protocol. Approximately 1 g of fecal material was collected from either the provided diaper (Parent's Choice, Walmart, Bentonville, AR) or directly from the skin of infants using a sterile spoon and collection tube (Sarstedt, Nümbrecht, Germany). Due to inconsistent electricity access, samples collected in rural Ethiopia were preserved in a ~1:4 ratio with RNAlater (Ambion), and frozen at −20° C within 6 days. All other fecal samples were collected and stored in sterile polypropylene containers (Sarstedt) and frozen at −20°C within 30 min of collection. Fecal samples were shipped on dry ice to the University of Idaho, and immediately frozen upon arrival at −20°C. Out of 413 mother–infant dyads initially enrolled in the study, a total of 406 infant fecal samples were collected.

2.3 | Infant fecal sample DNA extraction

Four hundred three infant fecal samples were successfully preserved and received by the University of Idaho (see Lackey et al., 2019, for detailed explanation of sample exclusion). DNA was extracted from 398 of the samples; the remaining five samples' DNA could not be extracted due to inadequate material or sample condition. Prior to extraction, samples were thawed at room temperature, and approximately 0.2 g placed into a sterile microcentrifuge tube with 500 μL of TE-50 (10 mM Tris–HCl, 50 mM EDTA, pH 8). The mixture was then vortexed until homogenous and frozen at −80°C until extraction. In cases where less than 0.2 g was available, 500 μL of TE-50 was directly added to the fecal material in the collection tube and vortexed until homogenous, and the suspension transferred into a separate sterile tube and frozen at −80°C until extraction. In preparation for extraction, samples were quickly thawed on a dry heat block and re-homogenized by vortexing. DNA was extracted using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) with an additional bead beating step using 0.1 mm diameter zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK) and a FastPrep FP120A-115 (Qbiogene, Carlsbad, CA). Additionally, 500 μL of TE-50 from the aliquot used to prepare the fecal samples and 500 μL of sterile water were extracted in parallel as negative controls. DNA was eluted with 200 μL of ATE buffer supplied in the Qiagen kit (10 mM Tris–Cl pH 8.3, 0.1 mM EDTA, 0.04% Sodium azide) and was subsequently stored at −80°C until amplification.

2.4 | Bacterial DNA amplification

Bacterial DNA in the infant fecal samples was amplified in a two-step reaction using primers for the V1–V3 hypervariable regions of the 16S rRNA gene. PCR was conducted using 96-well plates (USA Scientific, Ocala, FL). The reaction mix was comprised of 12.5 μL Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs®, Inc., Ipswich, MA), 0.25 μL each of forward and reverse primers (27F and 534R, respectively; Carrothers et al., 2015; Frank et al., 2008; Lackey et al., 2017), 2 μL template DNA, and 8 μL nuclease-free, sterile water to bring the first reaction volume to 23 μL. For each plate, 2 μL of nuclease-free water was used as a negative control; 2 μL of *Escherichia coli* DNA (221 ng/mL) was used as a positive control. DNA was then amplified in a Veriti thermal cycler (Applied Biosystems, Foster City, CA) with the following conditions: initial denaturation at 98°C for 30 s; then 15 cycles of denaturation at 98°C for 10 s, annealing at 51°C for 20 s, and extension at 72°C for 20 s. Samples were then removed from the thermal cycler and 2 μL of a uniquely barcoded primer with Illumina adapters was added to each sample. The plate was vortexed, centrifuged briefly, and returned to the thermal cycler within 10 min. Once replaced into the thermal cycler, the plate underwent the same denaturation and 15 cycles of amplification as described previously, with the exception of higher annealing at 60°C; followed by a 2-min hold at 72°C and final hold at 4°C until the plate was removed from the thermal cycler.

2.5 | DNA quality assessment and pooling

Positive and negative PCR products and a 1-kb ladder (Thermo Fisher Scientific, Grand Island, NY) were electrophoresed at constant voltage (80 V) on a 1% agarose gel in tris-acetate-ethylenediamine tetraacetic

acid buffer (TAE; 40 mM Tris, 20 mM acetic acid, 1 mM EDTA) for 30 min. DNA was stained using GelRed (10×, Biotium, Fremont, CA) and visualized using an UltraCam Digital Imaging System (BioRad, Hercules, CA). All PCR products were evaluated using high-resolution capillary electrophoresis on a QIAxcel Advanced System (Qiagen). Briefly, 2 μL of the PCR product was added to 8 μL QX DNA dilution buffer and run on a QIAxcel DNA Screening cartridge. Quality amplicons were those showing a peak at the correct amplicon size according to the hypervariable region of interest and low primer dimers (<100 bp). The Qubit® 2.0 Fluorometer and the Qubit® dsDNA High Sensitivity (HS) Assay (Thermo Fisher Scientific, Waltham, MA) were then used to quantify DNA (2 μL PCR product combined with 198 μL of Qubit working solution [working solution made as per manufacturer's instructions]). Composite pools of amplicons were created by combining 50 ng of each sample's PCR product. If a sample had <50 ng of DNA, an attempt was made to re-amplify the sample. If this was unsuccessful, the entire volume of PCR product was used. All 398 samples were retained for further analysis.

2.6 | Microbial DNA sequencing and analysis

Amplicon pools were submitted for further processing and sequencing at the University of Idaho's Institute for Bioinformatics and Evolutionary Studies Genomics Resource Core (IBEST GRC). Briefly, amplicon pools were size-selected using AMPure beads (Beckman Coulter, Indianapolis, IN) and quantified using the KAPA Biosciences Illumina library quantification kit and Applied Biosystems StepOne Plus real-time PCR system. Sequences were obtained with Illumina MiSeq (San Diego, CA) v3 paired-end 300-bp protocol for 600 cycles. Sequence reads were demultiplexed using the custom python application dbcAmplicons (https://github.com/msettles/dbcAmplicons). During preprocessing, barcodes were allowed ≤1 mismatch (hamming distance), and primers were allowed ≤4 mismatches (Levenshtein distance) as long as the final four bases of the primer perfectly matched the target sequence. Sequence reads without a corresponding barcode or primer sequence were excluded. Sequence reads were also trimmed of their primer sequences. Reads were then assigned into two separate R1 and R2 files for each sample using a custom python script (splitReadsBySample.py; https://github.com/msettles/dbcAmplicons/blob/master/scripts/python/splitReadsBySample.py). Sequence reads were then assessed for sufficient quality, trimmed, and filtered using DADA2 (Callahan et al., 2016). Only forward reads were used for the analysis, due to the small amount of overlap between forward and reverse reads following DADA2 trimming and filtering. The output from DADA2 ("amplicon sequence variants" [ASV]) were assigned to taxonomies using the Ribosomal Database Project (RDP) Bayesian classifier (Wang, Garrity, Tiedje, & Cole, 2007) and the SILVA 16S rRNA database version 123 formatted for DADA2 (Callahan, McMurdie, & Holmes, 2017; Quast et al., 2012; Yilmaz et al., 2013;). Samples with read counts <1,000 at the genus level (n = 21) were excluded from analysis, resulting in a sample size of 377.

2.7 | Subject characteristics and household composition

Infant and maternal characteristics (infant and maternal age, birth mode, and exclusive breastfeeding status) and household composition data were collected on participants. However, of the 377 fecal samples that were retained, only 360 maternal/infant dyads had complete characteristic and household composition data. Four of the dyads had missing data in regards to either birth mode or infant age, and detailed household demographic data were unavailable for the U.S. Hispanic population and from one infant in the rural Gambia population. All remaining (n = 360) infants' mothers provided complete and detailed household composition data, including number, age, sex, and relation to the focal infant of all children and adults currently living in the home. Age of mothers, focal infants, and all individuals present in the household were calculated to the exact day of birth when possible. In populations where it is uncommon to know exact birthdates, timing of the mothers' reproductive histories and local events were considered in order to carefully estimate ages in years (this applied to a small proportion of our Ethiopian participants). It is unlikely that mothers incorrectly estimated their infants' birth dates, given how little time had passed since their births (≤5 months; Ruiz et al., 2017). Ethics approvals in Sweden only permitted the collection of infant birth year and age rounded to the nearest week.

In order to investigate how IFM communities may be influenced by the presence or absence of members belonging to particular household composition categories, we calculated overall household size, the number of siblings, the presence/absence of the biological father, and a category we refer to as "extended family," which includes number of maternal and paternal aunts and uncles, maternal and paternal grandparents, and nonrelative female and male adults and juveniles for each household. To be considered a member of the household, individuals had to be currently residing in the home. Overall, household number was the summation of all individuals living in the household at the time of sampling, including the mother and focal infant.

2.8 | Statistical analyses

All statistical analyses were performed using the R software (version 3.4.1; R Core Team, 2017). Our analysis used unrarefied microbiome counts to avoid omitting valid data and to maintain maximum statistical power (McMurdie & Holmes, 2014). Bacterial richness and alpha diversity indices (Shannon-Wiener diversity index [Shannon's H] and Shannon evenness) were calculated using the ASV-level data and relative abundances of specific taxa were calculated using the sequence read counts following agglomeration at the genus level in phyloseq (McMurdie & Holmes, 2013). Shannon's H accounts for both abundance and evenness of bacterial genera present in a sample. Shannon's H is calculated by first dividing the individual genus abundance by the total abundance of all genera detected in the sample, and multiplying this ratio by the natural logarithm of the individual genus abundance; this process is repeated for all detectable genera in the sample, and the summation of all products results in H. Because measurements of participants within populations are not statistically

independent, linear mixed effects (LME) models (nlme package; Pinheiro et al., 2017) with random intercepts for population were used to assess potential relationships between household categories (number of siblings and number of extended family members) and IFM Shannon's *H*, Shannon evenness, and richness. The random intercept also takes advantage of partial pooling, meaning that intercept estimates for each population are partially based upon data from the other populations in the sample. Estimates for populations with relatively few data points will therefore tend to "shrink" toward the grand mean (Gelman & Hill, 2007). Each LME model also included fixed effects controlling for biological father presence (present/absent), focal infant age (weeks), birth mode (vaginal/cesarean), and exclusive breastfeeding status (exclusive/nonexclusive).

To investigate the potential influence that household composition had on IFM taxonomic composition, we implemented analysis of composition of microbiomes (ANCOM; Mandal et al., 2015). ANCOM uses a linear framework to statistically detect taxa whose composition varies across changing household composition, while controlling for other variables of interest. For any specific taxa, this method proceeds by computing log ratios of the count data, where the normalizing reference value is the abundance of all remaining taxa taken one at a time.

ANCOM was chosen for this analysis because it does not make distributional assumptions of the data, but instead carries out hypotheses tests via bootstrapped intervals. More importantly, ANCOM has been demonstrated to successfully maintain the desired false discovery rate (FDR). Weiss et al. (2017) provide a concise description of ANCOM along with comparisons to other available methods in the literature, designed for microbiome data. We implemented ANCOM with the FDR level set to 0.05. A genus was considered significantly varying in composition across an independent variable of interest at a W-statistic level of 0.7, meaning that the taxa composition was varied across the independent variable with respect to 70% of reference taxa; see Mandal et al. (2015) for details regarding the W-statistic. ANCOM was used to explore whether IFM composition at the genus level was associated with total number of siblings, sibling presence, number of extended family, extended family presence, overall household size, and father presence. All ANCOM tests controlled for the fixed effects of population, infant age (weeks), delivery mode (vaginal/cesarean), and exclusive breastfeeding status (exclusive/non-exclusive). While ANCOM provides a list of taxa that vary in composition across independent variables, it does not provide the directionality of these associations. In order to evaluate the directionality of relationships between household composition and relative abundances of specific taxa, we implemented a two-step follow-up regression method. First, the logarithm of the geometric mean of the total sequencing read counts of bacteria present in a sample is regressed on the independent and control variables. The residuals of this regression provide an estimate on the variation due to sampling depth (library effect). The second step regresses the logarithm of counts corrected by the previously estimated library effects on the desired independent and control variables. This method is a natural extension of that proposed in Kaul et al. (2017) in a two-population setting. Follow-up regression analyses considered unadjusted *p* values (<.05) as statistically significant, as these analyses were testing relationships between household categories and taxa previously identified as significant by ANCOM.

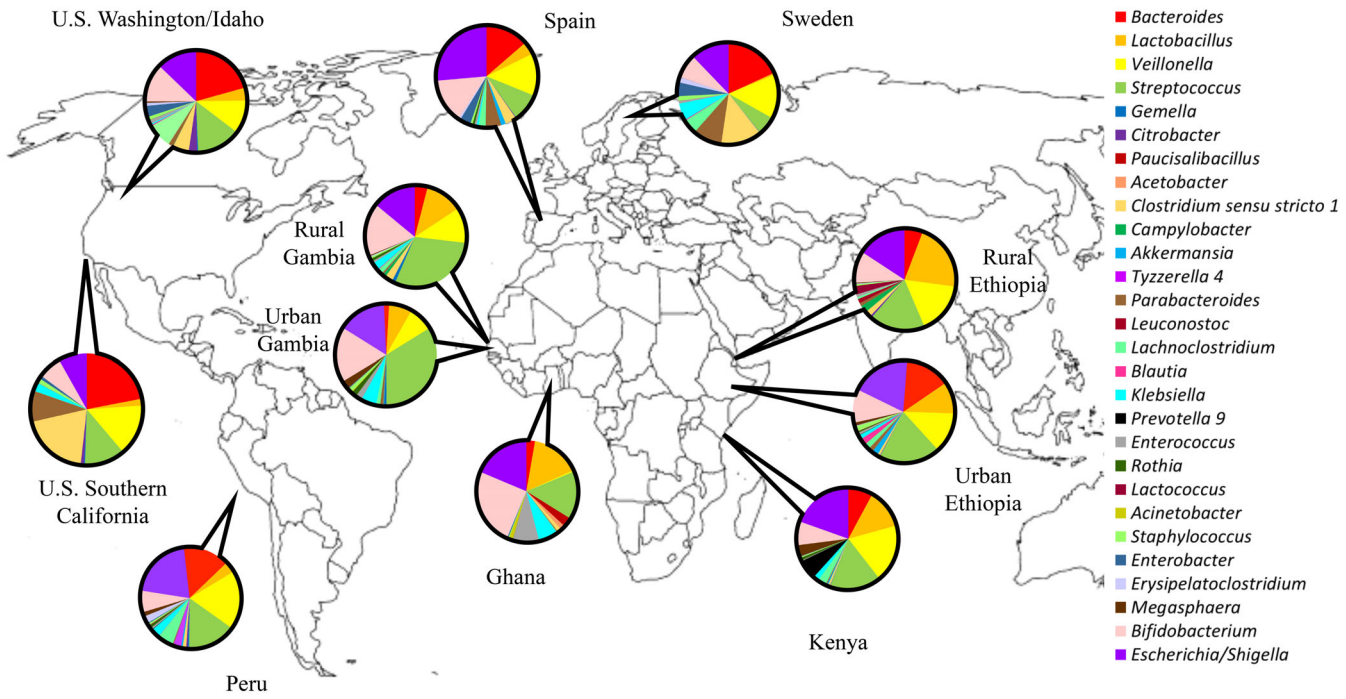

FIGURE 1 Top 10 most abundant infant fecal microbiome taxa from each population, aggregated to a list of 28 taxa. The sites depicted are as follows: United States–Washington/Idaho (*n* = 41), United States–Southern California (*n* = 12), Peru (*n* = 42), Spain (*n* = 37), Sweden (*n* = 23), rural Gambia (*n* = 38), urban Gambia (*n* = 38), Ghana (*n* = 32), rural Ethiopia (*n* = 40), urban Ethiopia (*n* = 32), and Kenya (*n* = 42)

3 | RESULTS

3.1 | Sequencing summary

The sequencing run for the 377 infant fecal samples generated 4,384,377 reads, with a mean (± standard deviation [*SD*]) of 11,630 ± 6,275 reads following initial processing using the DADA2 workflow. After additional filtering of any read that could not be classified to the genus level, the infant fecal dataset analyzed here contained 4,314,551 reads, with a mean (±*SD*) of 11,444 ± 6,198 reads and a range of 1,662–40,255 reads.

3.2 | Study populations and IFM characteristics

Infant ages across all populations ranged from 2.9 to 23.0 weeks (*M* = 9.2, *SD* = 3.1 weeks; excluding one infant each from the Kenyan and rural Gambian populations, due to missing data). Infants across the populations were 49% female (excluding one infant each from the rural Ethiopian, Kenyan, United States-Hispanic, and rural Gambian populations, due to missing data). Maternal ages ranged from 18 to 46 years (*M* = 27.5, *SD* = 6.1; excluding two mothers from the rural Gambian population, and one each from the urban Gambian and Kenyan populations).

In order to briefly characterize the IFM across the 11 populations, we present the 10 most relatively abundant bacterial genera in the infant feces collected from each study population, together comprising a total of 28 "aggregated" genera (Figure 1). All subsequent analyses in the study, comprising a sample of *n* = 360, were conducted on all genera detected in the IFM samples. Seventeen of the 28 aggregated genera were present in all 11 study populations; these included *Bacteroides*, *Lactobacillus*, *Veillonella*, *Streptococcus*, *Gemella*, *Citrobacter*, *Clostridium* sensu stricto *1*, *Parabacteroides*, *Lachnoclostridium*, *Klebsiella*, *Enterococcus*, *Rothia*, *Staphylococcus*, *Enterobacter*, *Erysipelatoclostridium*, *Bifidobacterium*, and *Escherichia/Shigella*. *Streptococcus* was the most relatively abundant genus observed in urban and rural Gambia, and urban Ethiopia. *Escherichia/Shigella* was the most prominent in the Kenyan and Spanish populations. Infant feces collected in Sweden, Peru, and the United States (Washington state/Idaho and Southern California) were predominated by *Bacteroides*. *Lactobacillus* and *Bifidobacterium* were the most prevalent genera in the rural Ethiopian and Ghanaian populations, respectively. *Leuconostoc* was included in the top-10 most abundant genera only in populations from the African continent, except in rural Gambia. All populations lacked the genus *Paucisalibacillus* except rural Gambia, Ghana, Sweden, and Spain. The genus *Acinetobacter* was observed in all populations except Kenya and the United States (Southern Californian Hispanic population). The genera *Campylobacter* and *Megasphaera* were present in all populations except Spain and the United States (Southern Californian Hispanic population). The genus *Prevotella 9* was only found in fecal samples from the urban Ethiopian, rural and urban Gambian, Kenyan, and Peruvian populations. Lackey et al. (2019) provide a more thorough characterization of IFM similarities and variation across these study populations.

TABLE 1 Household composition characteristics by population[a]

Population (abbreviation)	*n*	Mean infant age (weeks) ± *SD*	Proportion of infants delivered vaginally	Proportion of infants exclusively breastfed	Mean household size ± *SD* (range)	Mean number of siblings ± *SD* (range)	Proportion of infants with siblings present	Mean number of extended family ± *SD* (range)	Proportion of infants with extended family present	Proportion of infants with fathers present
Ethiopia rural (ETR)	39	10.07 ± 4.60	1.00	0.97	6.26 ± 1.82 (3–11)	2.54 ± 2.04 (0–8)	0.82	0.72 ± 1.38 (0–6)	0.28	1.00
Ethiopia urban (ETU)	32	8.31 ± 2.20	1.00	0.88	4.47 ± 1.50 (3–9)	0.59 ± 1.10 (0–4)	0.31	0.91 ± 1.06 (0–4)	0.59	0.97
The Gambia rural (GBR)	36	8.99 ± 2.40	1.00	0.97	6.19 ± 2.14 (4–13)	2.06 ± 1.98 (0–7)	0.72	1.53 ± 2.01 (0–8)	0.53	0.61
The Gambia urban (GBU)	38	8.88 ± 2.61	0.97	0.53	5.97 ± 1.88 (3–10)	1.84 ± 1.67 (0–5)	0.68	1.45 ± 2.06 (0–6)	0.50	0.68
Ghana (GN)	32	8.35 ± 2.85	0.81	0.34	5.66 ± 1.72 (3–11)	1.31 ± 1.12 (0–4)	0.69	1.75 ± 1.85 (0–7)	0.72	0.63
Kenya (KE)	40	10.34 ± 3.41	0.78	0.38	4.65 ± 1.61 (2–8)	1.45 ± 1.43 (0–5)	0.73	0.58 ± 1.20 (0–4)	0.23	0.63
Peru (PE)	42	8.61 ± 2.42	0.52	0.31	6.02 ± 2.26 (3–13)	1.00 ± 0.96 (0–4)	0.67	2.12 ± 2.56 (0–10	0.55	0.90
Spain (SP)	37	10.25 ± 3.52	0.92	0.62	3.24 ± 0.49 (3–5)	0.22 ± 0.42 (0–1)	0.22	0.03 ± 0.16 (0–1)	0.03	1.00
Sweden (SW)	23	6.87 ± 2.64	0.78	0.48	3.43 ± 0.73 (2–5)	0.48 ± 0.67 (0–2)	0.39	0.00 ± 0.00 (0–0)	0.00	0.96
United States–Washington/Idaho (USW)	41	9.67 ± 2.39	0.80	0.56	3.85 ± 1.11 (2–8)	0.85 ± 1.04 (0–5)	0.56	0.05 ± 0.31 (0–2)	0.02	0.95

[a]*n* = 360, USC population was excluded due to incomplete household composition data, and four additional observations omitted due to incomplete data amongst the control variables (age missing from one infant in GBR; birth mode missing from two infants in KE and one infant in ETR).

TABLE 2 Linear mixed effects model summaries of household composition and infant fecal microbiome alpha diversity and richness metrics[a]

	(a). Shannon-Wiener Diversity Index (*H*)				(b). Shannon evenness				(c). Richness		
Covariate	**Coefficient**	***SE***	***p***	**Covariate**	**Coefficient**	***SE***	***p***	**Covariate**	**Coefficient**	***SE***	***p***
Siblings	−0.22	0.25	.37	Siblings	−0.01	0.004	.17	Siblings	−0.05	0.52	.92
Extended family	0.15	0.24	.54	Extended family	0.003	0.004	.43	Extended family	−0.07	0.496	.89
Father presence	−0.55	1.03	.59	Father presence	−0.006	0.02	.73	Father presence	−0.43	2.16	.84
Infant age (weeks)	0.22	0.11	.05	Infant age (weeks)	0.0003	0.002	.87	Infant age (weeks)	0.48	0.23	.04
Delivery mode[b]	−0.67	1.01	.51	Delivery mode[b]	−0.02	0.02	.19	Delivery mode[b]	0.56	2.13	.79
Exclusive breastfeeding status[c]	−0.46	0.76	.54	Exclusive breastfeeding status[c]	−0.01	0.01	.42	Exclusive breastfeeding status[c]	0.08	1.60	.96
Intercept	13.88	1.84	.00	Intercept	0.37	0.03	.00	Intercept	38.17	3.89	.00
Standard deviation of random intercept = 2.05				Standard deviation of random intercept = 0.05				Standard deviation of random intercept = 4.46			

[a]*n* = 360, USC population was excluded due to incomplete household composition data, and four additional observations omitted due to incomplete data amongst the control variables (age missing from one infant in GBR; birth mode missing from two infants in KE and one infant in ETR).
[b]Delivery mode is a binary variable coded as: cesarean birth = 0, vaginal birth = 1.
[c]Exclusive breastfeeding status is a binary variable coding infant diet as: exclusively breastfed = 0, diet which may include a mixture of human milk, formula, other liquids and semi-solid foods = 1.

3.3 | Household composition and IFM diversity and richness

For the 360 infants for which we had household composition data, household sizes ranged between 2 and 13 individuals across all populations (*M* = 5.04, *SD* = 1.98). A minimum of 0 and maximum of 8 total siblings in a household were observed (*M* = 1.27, *SD* = 1.52). The range of extended family individuals residing in the households ranged from 0 to 10 (*M* = 0.94, *SD* = 1.70). Table 1 presents summary statistics on infant age, proportion of infants delivered vaginally, proportion of infants exclusively breastfed, household size, siblings, extended family, and father presence.

LME models assessed potential relationships between the selected household categories (number of siblings and number of extended family members) and IFM Shannon's *H*, Shannon evenness, and richness. Overall household size was not included in these models, because, as we would expect, it is highly correlated with the other household variables. LME modeling indicated no statistically significant effects of household composition on IFM diversity, evenness, or richness (Table 2a–c). The summary output of the same LME models including diversity metrics computed from rarefied, as opposed to non-normalized, microbiome count data can be found in Table S1; we observed essentially no differences between these two sets of models.

3.4 | Household composition and IFM taxonomic composition

We next, via ANCOM, examined whether household composition was associated with the relative abundance of specific genera (Table 3). Both sibling presence and number of siblings were associated with variation in the relative abundance of *Lactobacillus*. Presence of extended family in the home corresponded to variation in the relative abundances of *Klebsiella*, *Clostridium* sensu stricto *1*, and *Enterobacter*. Number of extended family was associated with variation in the relative abundance of *Klebsiella*. Overall household size was associated with variation in the relative abundances of *Lactobacillus*, *Enterobacter*, *Clostridium* sensu stricto *1*, and *Klebsiella*.

We then implemented a two-stage follow-up regression to evaluate the directionality of potential linear relationships between the

TABLE 3 Infant fecal microbiome genera that are significantly related to variation in household composition (ANCOM)[a]

	Lactobacillus	***Klebsiella***	***Clostridium* sensu stricto 1**	***Enterobacter***
Number of siblings	✓			
Sibling presence	✓+			
Number of extended family		✓+		
Extended family presence		✓+	✓	✓
Household size	✓	✓+	✓	✓

[a]*n* = 360, USC population was excluded due to incomplete household composition data, and four additional observations omitted due to incomplete data amongst the control variables (age missing from one infant in GBR; birth mode missing from two infants in KE and one infant in ETR). Check marks indicate a significant relationship between the household composition variable and the taxa as identified via ANCOM. For all analyses, the FDR was set at 0.05 and the W-statistic at 0.7. Positive signs indicate that the association between household categories and IFM genera was positive and statistically significant in the follow-up regression analyses ($p < .05$).

discrete household categories and their corresponding influential taxa (Table 3). There were significant positive linear relationships between *Lactobacillus* and sibling presence (ß = 0.54, p = .04) and *Klebsiella* and extended family presence (ß = 0.71, p = .01), number of extended family members (ß = 0.27, p < .01), and household size (ß = 0.17, p = .01). Relationships between household composition and the relative abundances of their corresponding influential taxa are illustrated in Figures S1–S5, largely reflecting the trends found in the follow-up regression method. Of important note is that while ANCOM found all the previously described relationships to be statistically significant, cases where the follow-up regression analyses did not also find statistical significance simply indicates that those correlations are nonlinear.

4 | DISCUSSION

We examined the relationships between household composition and the IFM, testing the hypotheses that: (a) number of siblings, number of extended family, and household size would be associated with increased IFM diversity, evenness, and richness and (b) number of siblings, number of extended family, and household size would predict variation in the relative abundances of members of commensal bacteria in the IFM. To our knowledge, our study is the first to investigate whether the number of family members other than siblings, such as grandparents, aunts/uncles, cousins, and biologically unrelated coresidents, may correspond to differences in the IFM profile.

In reference to the overall characterization of the IFM in our study, we observed that 17 of the top 28 aggregated abundant genera were present in all study populations, including *Bacteroides*, *Lactobacillus*, *Veillonella*, *Streptococcus*, *Gemella*, *Citrobacter*, *Clostridium* sensu stricto *1*, *Parabacteroides*, *Lachnoclostridium*, *Klebsiella*, *Enterococcus*, *Rothia*, *Staphylococcus*, *Enterobacter*, *Erysipelatoclostridium*, *Bifidobacterium*, and *Escherichia/Shigella*. IFM samples collected from our study populations were dominated by *Streptococcus*, *Lactobacillus*, *Veillonella*, *Bacteroides*, and *Escherichia/Shigella*, as reported elsewhere by our group (Lackey et al. 2019) and in line with previous findings (Bäckhed et al., 2015; Davis et al., 2017; Milani et al., 2017; Murphy et al., 2017). A more thorough characterization of IFM composition across our study cohorts can be found in Lackey et al. (2019). More germane to the relationship between infants' social environments and IFM composition, taxa identified by ANCOM to be related to variation in household composition were present in IFM samples from all populations included in the study, namely: *Lactobacillus*, *Clostridium* sensu stricto *1*, *Enterobacter*, and *Klebsiella*. These four taxa were among the top 12 most abundant aggregated genera across all study populations and thus make a substantial contribution to the overall community composition. Lackey et al. (2019) provide further discussion regarding similarities and differences in IFM across populations, with respect to geographic locations and other characteristics.

LME results indicated no significant relationships between household composition (number of siblings and number of extended family members) and the diversity, evenness, or richness of the IFM. As these models treat population as a random effect with partial pooling, for populations with small household data ranges (e.g., Sweden and Spain), estimates of the intercept are partially based upon data from other populations. This method of analysis can improve estimates for groups with small sample sizes; however, partial pooling might mask population-specific effects.

Although potential relationships between household composition and IFM alpha diversity could vary by population, given the small ranges of household composition categories amongst many of the populations in our dataset (e.g., number of siblings in Western populations) and our low population sizes, we were not able to model these relationships including an interaction with population. However, with availability to larger population-level sample sizes, this will be important to explore in future work, as cross-cultural variation in economic demands, subsistence strategies, gender norms, and childcare practices likely modifies both the extent to which coresidents engage in external environments and how frequently they physically interact with and care for the focal infant. These two patterns undoubtedly change an individual's capacity to be a vector of socially mediated bacteria. Additionally, coresidents may exert more of an influence on the overall taxonomic profile of the IFM, rather than alpha diversity and richness.

Our results indicated that presence of siblings was associated with an increased relative abundance of *Lactobacillus* in the IFM, consistent with that of Penders et al. (2013) study, who observed that number of siblings is correlated with an increase in the colonization rate of *Lactobacillus* in the IFM. Additionally, we found that extended family presence was significantly associated with the relative abundance of *Clostridium* sensu stricto *1*, although our follow up linear regression analysis did not find a significant reduction or increase in relative abundance of *Clostridium* sensu stricto *1* when extended family members resided in the household. Penders et al. (2006) and Adlerberth et al. (2007) noted that the IFM was colonized early with *Clostridium* species in the absence of siblings. Perhaps, although we cannot directly evaluate the colonization pattern, we are seeing a similar trend where the presence of extended family inhibits proliferation of *Clostridium* sensu stricto *1* in the IFM. Although it is difficult to infer genus-level functionality, the presence of *Lactobacillus* in the infant GIM appears to be protective against atopic outcomes and autoimmune disease (Johansson, Sjögren, Persson, Nilsson, & Sverremark-Ekström, 2011; Sjögren et al., 2009; for review: Kosiewicz, Zirnheld, & Alard, 2011). Additionally, a species of *Clostridium* (*Clostridium difficile*) has been shown to increase risk for childhood atopies and frequency of postnatal hospitalization (Penders et al., 2006; Penders et al., 2013; van Nimwegen et al., 2011).

Three primary limitations of the study make it difficult to more fully address questions regarding socially mediated transmission of bacteria in humans and its associated evolutionary implications. First, household composition alone is not the most predictive indicator of how many people regularly physically interact with and care for the infant and thus likely does not accurately represent the potential for social transmission of bacteria. To truly assess the size and intensity of infants' social networks, we need quantifiable behavioral observations of infant behavior and interactions with others in a naturalistic

setting, as demonstrated in studies amongst hunter-gatherers and horticulturalists in the Central African Republic (Meehan et al., 2018) and nonhuman primates (Amato et al., 2017; Moeller et al., 2016; Tung et al., 2015). For instance, Meehan et al. (2018) showed that the size of caregiving networks and the frequency of allomaternal care infants received, not just who was present in the household, were associated with increased bacterial diversity and evenness in mothers' milk. Second, the ability to examine the IFM profile relative to the fecal microbiome profiles of other household members would better inform our understanding of the potential for horizontal transfer of bacteria between individuals in the household, as demonstrated among family groups in human and nonhuman animal studies (Koch & Schmid-Hempel, 2011; Lax et al., 2014; Tung et al., 2015). Third, we have yet to examine how household composition may influence the maternal milk microbiome of the mothers in our study; these analyses are forthcoming. For breastfed infants, milk is thought to lay down the foundations of the infant GIM, in addition to other factors such as gestational age, birth mode, and antibiotic exposure (for review, see Milani et al., 2017). It may be more important to examine how the whole of the social network, not just household composition, of the mother–infant dyad may influence milk microbial profiles.

Additionally, a methodological consideration is that the fecal samples were thawed and refrozen once prior to extraction. Although multiple freeze–thaw cycles were shown to alter bacterial community composition, Gorzelak et al. (2015) reported no significant changes in bacterial composition up to three freeze–thaw cycles.

This study, however, contributes to our understanding of the evolutionary implications of socially mediated mutualistic bacteria. The "old friends" hypothesis (Rook, 2010) provides a framework by which we may understand potential relationships between the microbiome, its environmental and social vectors, and infant immunity. This hypothesis maintains that pre- and postnatal exposure to microbes with which the human species has a long coevolutionary history serves to prime infant immunoregulation (Bloomfield et al., 2016; Rook, Bäckhed, Levin, McFall-Ngai, & McLean, 2017). Byproducts of group living and cooperative breeding, such as heightened social physical contact and number of interactors, may facilitate socially mediated transmission of microbiota, which in turn contributes to bolstering the neonate immune system within the social network. Thus, cooperative breeding and sociality more generally may enhance individual fitness by facilitating immunity in early development, in addition to other benefits of group living and cooperative breeding, such as decreased predation risk, increased foraging efficiency, enhanced defense of resources, (Alexander, 1974; Trivers, 1985; van Schaik, 1983; Wilson, 1975; Wrangham, 1980), offspring nutritional provisioning (Meehan, Helfrecht, & Malcom, 2016), and enhanced socialization of young (Pereira & Fairbanks, 2002). More specifically, that some human-adapted bacteria are potentially commensal or even mutualistic may offset demonstrated costs of group living, such as increased exposure to pathogens (Ezenwa, Ghai, McKay, & Williams, 2016). This idea has been suggested previously with respect to nonhuman animals (Archie & Theis, 2011; Lombardo, 2008), but holds especially important implications for humans, cooperative breeders who to a varying extent, rely on alloparental networks to raise our offspring (Hrdy, 2009; Meehan, 2005).

According to the logic of the "old friends" hypothesis, we would expect the number of household members cohabitating with the focal infant to promote the diversity, evenness, and richness of the IFM. Our results did not indicate this effect of household composition, although, as mentioned previously, household composition alone may not accurately represent the infant social network, particularly in non-Western contexts. With respect to the relationship between household composition and IFM community structure, our results reflect previous findings indicating that siblings and other coresidents seem to be associated with increased prevalence of "old friends" (e.g., *Lactobacillus*). Enhancement of diversity and richness of the IFM, or more broadly, their GIM, may not be the primary advantage of socially mediated microbial transmission, but rather, the transmission of specific beneficial bacterial taxa, the likelihood of which would be increased by promotion of bacterial diversity (Azad et al., 2013). Furthermore, as Tung et al. (2015) observed in baboons, certain bacterial taxa may be more susceptible to social transmission than others. The potential for socially mediated bacteria within human families may vary across different environmental and cultural contexts. Future work will continue to incorporate a cross-cultural perspective, examine the functionality of socially mediated GIM taxa, and implement more quantitative and expansive measures of the social and alloparental networks of mother–infant dyads, to more closely explore how the infant GIM is shaped by the nature of their social worlds.

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SUPPORTING INFORMATION

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