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The development and ecology of the Japanese macaque gut microbiome from weaning to early adolescence in association with diet

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Abstract

Previously we have shown that the Japanese macaque gut microbiome differs not by obesity *per se*, but rather in association with high fat diet feeding. This held true for both pregnant dams, as well as their one-year old offspring, even when weaned onto a control diet. Here we aimed to examine the stability of the gut microbiome over time and in response to maternal and post-weaning high fat diet (HFD) feeding from 6 months of age, and at 1 and 3 years of age. In both cross-sectional and longitudinal specimens, we performed analysis of the V4 hypervariable region of the 16S rRNA gene on anus swabs collected from pregnant dams and their juveniles at age 6 months to 3 years (n=55). Extracted microbial DNA was subjected to 16S amplicon-based metagenomic sequencing on the Illumina MiSeq platform. We initially identified 272 unique bacterial genera, and multidimensional scaling (MDS) revealed samples to cluster by age and diet exposures. Dirichlet multinomial mixture modeling of microbiota abundances enabled identification of two predominant enterotypes to which samples sorted, characterized primarily by *Treponema* abundance, or lack thereof. Approximating the time of initial weaning (6 months), the Japanese macaque offspring microbiome underwent a significant state type transition which stabilized from 1 to 3 years of age. However, we also found the low abundance *Treponema* enterotype to be strongly associated with HFD exposure, be it during gestation/lactation or in the post-weaning interval. Examination of taxonomic co-occurrences revealed samples within the low

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Competing interests

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Treponema cluster were relatively permissive (allowing for increased interactions between microbiota) whereas samples within the high *Treponema* cluster were relatively exclusionary (suggesting decreased interactions amongst microbiota). Taken together, these findings suggest that Treponemes are keystone species in the developing gut microbiome of the gut, and susceptible to HFD feeding in their relative abundance.

Keywords

microbiome; macaque; high fat diet; early development

Introduction

The developmental origins of health and disease (DOHaD) suggests that adverse conditions in early life contribute to later-in-life metabolic disease (Barker, 1990, 1995; Barker, 2004; Eriksson et al., 2001; Roseboom, de Rooij, & Painter, 2006; Schulz, 2010). We have previously demonstrated in both humans and non-human primates that epigenetic, metabolic, and microbial disruptions both co-occur with exposure to a maternal high fat diet, cannot be completely ameliorated with postnatal weaning onto a control diet, but do diminish when obese dams are reverted to a control diet prior to pregnancy (Aagaard-Tillery et al., 2008; Chu et al., 2016; Cox, Williams, Grove, Lane, & Aagaard-Tillery, 2009; Ma et al., 2014; Pace et al., 2018; Suter et al., 2011; Suter et al., 2014; Suter, Chen, et al., 2012; Suter, Sangi-Haghpeykar, et al., 2012; Suter, Takahashi, Grove, & Aagaard, 2013). Interestingly, dysbiosis of the gut microbiome has similarly been shown to occur in temporal association with metabolic disease in adults (Dao et al., 2016; Delzenne, Neyrinck, & Cani, 2011; Devaraj, Hemarajata, & Versalovic, 2013; Everard et al., 2013; Larsen et al., 2010; Ley, 2010; Qin et al., 2012; Tilg & Kaser, 2011; Turnbaugh et al., 2009; Turnbaugh, Bäckhed, Fulton, & Gordon, 2008; Turnbaugh et al., 2006; Turnbaugh & Gordon, 2009); however, it is unclear if the microbiome is cause or consequence of these associations. Therefore, understanding the developmental and ecological alterations that occur within the gut microbiome from early life into adolescence may have the potential for identifying and developing interventions aimed at curbing the rate and occurrence of adult onset obesity and related metabolic disease.

With this in mind, precisely when and how the offspring gut microbiome is seeded and maintained remains unclear at present. While it is evident that while the gut microbiome varies by days and weeks of post-natal age (Chu et al., 2017; Koenig et al., 2011; Wu et al., 2011; Yatsunenko et al., 2012), diet and timing of feeding has been demonstrated to significantly alter both the structure and function of the gut microbiome (David et al., 2013). Furthermore, examination of the gut microbiome in first year of life suggests a relatively dynamic environment with several key transitions, albeit the relative impact of dietary components versus cessation of breastfeeding is still unknown (Bäckhed et al., 2015). To this end, we have previously demonstrated that exposure to a maternal high fat diet through gestation and lactation persistently alters the offspring microbiome in our primate model, notably with a diminished abundance of *Campylobacter* spp. (Ma et al., 2014). The use of synbiotics failed to persistently ameliorate the dysbiosis resulting from maternal high fat diet

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exposure, and does not prevent the re-occurrence of dysbiosis when challenged up to 2.5 years after initiation of a post-weaning control diet (Pace et al., 2018).

Given the evidence to date suggesting that the primate gut microbiome community structure and its function is persistently influenced by a maternal HFD, understanding the developmental and ecological alterations in the offspring gut microbiome over time and under distinct dietary feeding is of high significance and importance. Identifying key taxa and their metabolic functions which are under the influence of the maternal diet would retain the promise of identifying impactful interventions with the potential to mitigate the footprint left by a maternal high fat diet. Thus, we aimed to examine the ecology of the offspring gut microbiome from early life through adolescence in a primate model of maternal high fat diet and obesity using 16S-amplicon based metagenomic sequencing and analytic modeling in a cohort of dams and their offspring sampled at 6 months, 1 year, and/or 3 years of age.

Methods

Study design

This is a longitudinal and cross-sectional analysis of non-human primate dams and their offspring (Figure 1). The use of *Macaca fuscata* by our group of investigators has been previously described (Aagaard-Tillery et al., 2008; Cox et al., 2009; Ma et al., 2014; McCurdy et al., 2009; Pace et al., 2018; Suter et al., 2011; Suter, Chen, et al., 2012; Suter, Sangi-Haghpeykar, et al., 2012; Suter et al., 2013). Animals were socially housed within indoor/outdoor enclosures at the Oregon National Primate Research Center (ONPRC), meaning that for the duration in which they are co-housed animals their diets are shared. However, when their diets are switched (for example, post-weaning) then their social constructs will accordingly vary. Briefly, Japanese macaque dams were mated while consuming either a control (14% fat from soybean oil, Fiber-Balanced Monkey Diet 5052, Lab Diet, St. Louis, MO) or isocaloric high fat (36% diet from porcine and poultry fat, corn and fish oil, TAD Primate Diet – 5LOP, Test Diet, St. Louis, MO). Additionally, the high fat diet (HFD) group were supplemented with calorically dense treats (consisting of Glaxo powder/TAD pellets, peanut butter, honey, banana, and cornstarch). These diets continued through nursing resulting in offspring exposed to the maternal diet throughout gestation and lactation. After weaning (at approximately 6 to 7 months of age), offspring were either maintained on the maternal control diet (designated control/control) or weaned onto a control post-natal diet following maternal HFD feeding during gestation and lactation (high-fat/control). Administration of antibiotics preceding sample collection by 1 month did not occur in for any animal within the study. All methods were carried out in accordance with IACUC guidelines and regulations, and all experimental protocols were approved by the IACUC at ONPRC and Baylor College of Medicine. Notably, our use of both cross-sectional and longitudinal specimens reflects our overarching aims to minimize sedation to both dams and young offspring, and thus specimens were only collected when other *a priori* approved and scheduled clinical research procedures or necropsy were undertaken. All aspects of our research complied with the American Society of Primatologists Principles for the Ethical Treatment of Non-Human Primates.

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Gut microbiome sampling

When animals underwent metabolic testing (*e.g.* glucose-tolerance test or DEXA scan), Cesarean delivery (dams), or necropsy (36 month juveniles), anus samples comprised of stool from the high anus were collected using Catch-All Swabs (Epicentre, Madison, WI). This was primarily a cross-sectional study with a small subset of longitudinal sampling at between the 6- and 13-month timepoint. Swabs were vigorously swirled in MoBio PowerBead tubes (Qiagen, Germantown, MD), and samples were stored at −80°C prior to extraction. Microbial DNA was isolated using the MoBio PowerSoil protocol. Isolated DNA was subjected to 16S rDNA sequencing.

16S rRNA-amplicon based metagenomic sequencing and data processing

55 samples were sequenced on the Illumina MiSeq platform (2×250bp) of the 16S rRNA gene (V4 hypervariable region) at the Center for Microbiome and Metagenomic Research (CMMR) at BCM. The following primers were used: forward primer – 5’-GTGCCAGCMGCCGCGGTAA-3’; reverse primer – 5’-GGACTACHVGGGTWTCTAAT-3’. Raw reads were demultiplexed using idemp (https://github.com/yhwu/idemp). The demultiplexed data then had primers/adapters removed with cutadapt (Martin, 2013), and was further quality filtered and split into paired and unmatched reads with Trimmomatic (Bolger, Lohse, & Usadel, 2014). Quality filtered paired reads were then imported and processed with DADA2 (v1.6) (Callahan et al., 2016) in R (v3.4.3) (https://www.r-project.org/). Sequences were manually examined for drop off in sequencing quality and subsequently the forward and reverse reads were quality filtered and uniformly trimmed using the filterAndTrim() command. Error rates for both the forward and reverse reads were learned using the default settings. Sequence variants were inferred after sequence dereplication and paired reads merged to generate the amplicon sequence variants (ASVs). ASVs longer or shorter than the expected amplicon size were filtered out. Chimeric ASVs were identified using the command removeBimeraDenovo() using the consensus method. ASVs were assigned taxonomy with the assignTaxonomy() function using RDP’s naive Bayesian classifier against the provided Silva reference/training database (silva_nr_v128_train_set.fa.gz), with species level assignments also made using the addSpecies() function against the provided Silva species training data (silva_species_assignment_v128.fa.gz). The final ASV tables containing the abundance of each ASV in every sample were then imported into the phyloseq (v1.23.1) (McMurdie & Holmes, 2013) R package for downstream analysis. ASV tables were then filtered to remove non-Bacterial ASVs, including mitochondria and chloroplast, and unclassified Bacterial ASVs, as well as to remove samples with subsequent zero ASV counts.

Dirichlet multinomial mixture (DMM) modeling

The R package DirichletMultinomial(v1.20.0) (Holmes, Harris, & Quince, 2012) was used to describe the variability in the microbiome data and cluster samples into enterotypes based on the genus level ASV table. Model fit was determined based on the minimum Laplace goodness of fit.

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Statistical analysis

Samples were rarefied to 19,944 reads for multidimensional scaling (MDS) and alpha diversity analyses using the phyloseq rarefy_even_depth function. Statistical analysis of beta diversity was performed with permutational multivariate analysis of variance (PERMANOVA) using the vegan (v2.5–4) adonis function and 999 permutations on the distance matrices, with either the variable of interest added to the model or with animal identity and nested diet entered as a factor into the models before adding other variables. To examine alpha diversity and taxa by age at the genus level, QIIME was utilized (Caporaso et al., 2010). Alpha diversity metrics examined included observed ASVs, Shannon's diversity, Simpson's diversity, Good's Coverage, and Chao1. Statistical analysis of alpha diversity metrics was performed in Graphpad Prism (v7.0a, La Jolla, CA). Hierarchal clustering of taxa at the genus level was performed using Spearman's correlation available from Morpheus (https://software.broadinstitute.org/morpheus) was utilized. Prior to analysis of hierarchal clustering, genus level taxonomy below 0.01% was removed along with taxa not identified to the genus level. Examination of taxonomic differences were performed using linear discriminant analysis (LDA) of effect size (LEfSe) (Segata et al., 2011). Alphas were set to 1 for Kruskal-Wallis and Wilcoxon analysis with an LDA score of 2.0 and utilization of all-against-all for multi-class analysis. Post-analysis, false discovery rate (FDR) correction was performed using R p.adjust. Significant taxa (FDR-corrected $p<0.05$) resulting from this analysis are displayed. LEfSe was performed on full genus level taxonomy tables generated from ASVs through QIIME (Caporaso et al., 2010).

Differential taxonomic features based on the DMM clusters were identified via DESeq2(v1.18.1) (Love, Huber, & Anders, 2014). PICRUSt2 (Langille et al., 2013) was used to predict functions for ASVs and ascribe taxonomic contributions to inferred functional pathways. Briefly, ASV sequences were placed into the PICRUSt2 reference phylogeny, followed by the mp method for hidden-state prediction. The final outputs (metagenome predictions and predicted pathway abundances and coverages – stratified and non-stratified) were then exported for further analysis using R. Except where noted, all statistical analyses were performed using R (version 3.4.3) and/or GraphPad Prism (GraphPad Software Inc., La Jolla, CA). The R packages factoextra (v1.0.5), pheatmap (v1.0.8), vegan (v2.5–2) (Oksanen, Blanchet, & Friendly, n.d.), phyloseq (v1.23.1) (McMurdie & Holmes, 2013), and ggplot2 (v3.0.0) (Wickham, 2009) were used to perform and visualize cluster analyses and ordinations.

Data availability

The 16S amplicon-based metagenomic sequence data generated from this analysis has been deposited in the Sequence Read Archive (SRA) under bioproject ID PRJNA508806.

Results

16S rRNA amplicon-based metagenomic sequencing

A total of 55 samples were collected over a period of four years, with each subject sampled at least once but not necessarily at all time points (due to timing of scheduled procedures and necropsy; Fig. 1). A total of 2,012,245 filtered reads were obtained for all samples (average

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of 36,586 reads per sample) with minimum and maximum read counts of 26,592 and 45,089, respectively (Supp. Table 1). There was no difference in the number of reads based on diet, or when samples were stratified by age groups (Table 1). Additionally, we utilized Good's Coverage as a measure of sequence quality, which estimates the percentage of species represented in a given sample. For all samples, >99% of taxa were represented in the sample and thus indicating that high quality sequencing data had been generated among all subjects and their specimens (Supp. Table 1). Additionally, there were no significant differences in Good's Coverage between diet or age groups (Table 1). From these data we identified 3,173 amplicon sequence variants (ASVs), corresponding to 272 unique genera which were comparable by animal age and diet allocation with respect to both the number of 16S sequence reads generated and their quality.

Microbial community structure is associated with maternal diet and age

Upon initial examination of the microbial community and its composition, hierarchical clustering (Spearman's correlation) demonstrated that samples were predominantly structured by age and diet (Fig. 2A). To confirm these findings in an independent analysis, we performed multidimensional scaling (MDS) and found that the offspring gut microbiome was structured by diet across all samples (Bray-Curtis, PERMANOVA F-model=1.96, R^2=0.04, p=0.019) (Fig. 2B). Furthermore, the data demonstrated that post-natal age was also a driver of alterations in the gut microbiome (Bray-Curtis, PERMANOVA F-model=3.97, R^2=0.19, p=0.001)(Fig. 2C) with separate clustering of maternal, 6 month juvenile, 13 month juvenile, and 36 month juvenile samples by both weighted (Fig. 2C) and unweighted metrics (Supp. Fig. 1). These differences were not attributable to maternal age, parity, pre-pregnancy weight, housing, nor body composition as there were no significant differences in these demographics between the offspring cohorts (Supp. Table 2).

Since our cohort retained both cross-sectional and longitudinally sampled animals, we similarly analyzed our microbial community and its composition be inputting the animal identification first into the PERMANOVA modeling (see Methods). In doing so, animal identification (with its nested diet) and age were retained as a significant drivers of the alterations in the gut microbiome after removing non-significant interaction terms (animal identification and nested diet: F model 1.29, R^2 0.67, p=0.006; animal age: F-model 7.386, R^2 0.104, p=0.001). Similarly, the DMM cluster (see further below) was retained as significant (F-model of 4.07, R^2 0.07, p=0.001).

Examination of alpha diversity revealed significant differences based on post-natal age (Kruskal-Wallis, Dunn's corrected p<0.05 for Chao1 and observed ASVs, p>0.05 for Shannon and Simpson, Dunn's corrected), with 6-month old juveniles observed to have a lower alpha diversity compared to the older juveniles (p<0.05) and adults when utilizing Dunn's multiple comparisons test (Supp. Fig. 2). This difference in alpha diversity at 6 months of age was temporally associated with observed decreases in *Bifidobacterium* which occur post-weaning. Specifically, we found a significant decrease in *Bifidobacterium* after 6 months (Kruskal-Wallis, p=0.004; Dunn's post-test p<0.05), which would be anticipated to be attributed more to the absence of maternal breastfeeding and milk consumption rather than the addition of non-milk dietary constituents. Additionally, we observed alterations in

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alpha diversity when comparing control and high fat diet exposures with lower alpha diversity associated with high fat diet (all samples, $p<0.05$ by Mann-Whitney for Chao1, Observed ASVs, and Shannon). With our results demonstrating that the gut microbiome could be stratified by age and diet, we next sought to employ Dirichlet multinomial mixture (DMM) modeling to examine if enterotypes and transition states could be identified within the macaque gut microbiome.

Dirichlet multinomial mixture modeling reveals two enterotypes that shift with dietary changes

DMM modeling of microbiota abundances revealed that the offspring's samples were partitioned into two dominant community states, or enterotypes (Fig. 3A). Both enterotypes retained high abundances of *Prevotella* (Wilcoxon rank sum test, U=282.0, p=0.13), with state type categorization being significantly associated with variations in the relative abundance of *Treponema* spp. (Wilcoxon rank sum test, U=741.0, p=3.79e-10, FDR adjusted p=1.03e-7). When we allowed the taxonomy to further define our clusters in an unbiased fashion, we found that state types were categorized into two enterotypes as a function of (cluster A) observed high abundance of *Treponema* and (cluster B) observed low abundance of *Treponema*. A majority of samples were classified within the high *Treponema* abundance enterotype (n=31, ~56%), with the remaining samples in the low *Treponema* abundance enterotype (n=24, ~44%). Although with a lower relative abundance, we did find *Ruminococcus* spp. significantly co-occurring in cluster A rather B (Fig. 3A, Wilcoxon rank sum test, U=491, p=2.97e-5). Intriguingly, we observed that the DMM modeled enterotypes explained less of the variation in the microbial taxonomic abundance than post-natal age, but more than that explained by variation in maternal diet alone (Bray-Curtis, PERMANOVA F-model=7.69, R^2=0.11, p=0.001) (Fig. 3B) indicating shifts in enterotypes likely occur over time, but maternal diet has a persistent influence. Additionally, examination of alpha diversity by four independent measures (Observed, Shannon, Chao1, and Simpson) revealed the enterotypes to be significantly different (Mann-Whitney test, $p< 0.0001$), with the high *Treponema* abundance enterotype observed to have higher alpha diversity (Supp. Fig. 2). Given these observations in taxonomic structure and community variation by age and maternal diet, we next examined genus-level taxonomy with differential abundance using LEfSe.

Enterotypes are differentially enriched for predicted metabolic pathways

As enterotypes appeared to differ with respect to dietary exposures, we next utilized PICRUSt2 to infer the functional genetic potential of the macaque community state types. Altogether we identified 86 predicted pathways that were differentially enriched based on DMM cluster as determined via LEfSe (Fig. 4). The high *Treponema* abundance enterotype was enriched for 52 pathways, whereas the low *Treponema* abundance enterotype was enriched for 34 pathways (Fig. 4) when analyzed by LEfSe with an FDR correction. Within these enriched pathways, filtering taxa based on a contribution greater than the mean abundance revealed that, at least by 16S generated measures, as few as 51 taxa could be potentially driving these predicted functional distinctions (Fig. 4). Within the low *Treponema* abundance enterotype, we observed enrichment of fatty acid elongation. Within the high *Treponema* abundance enterotype, we instead observed an enrichment of pathways

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for the short chain fatty acid butanoate as well as an enrichment of preQ0 (prequeosine-0) biosynthesis.

Species co-occurrence modeling

We next sought to determine if microbial ecological networks and their landmark species might vary when comparing the high and low *Treponema* clusters identified by DMM. We hypothesized that the relative abundance of *Treponema* defining these clusters might not just influence community composition and function, but also affect the permissiveness of microbial interactions. When separated by high *Treponema* or low *Treponema* cluster, we found that the low *Treponema* group appeared more permissive with a 76% positive co-occurrence between genera (Fig. 5). Conversely, we observed a 46% positive co-occurrence in the high *Treponema* group (Fig. 5), a finding which was accompanied by a lower number of co-occurrences ($n = 124$) when compared to the low *Treponema* ($n = 335$). These alterations in co-occurrence were significant (Fisher's Exact Test, $p=5.5^{-8}$, odds ratio 0.28). Altogether, this data is consistent with our prior observations and collectively suggests that high *Treponema* may be a more restrictive enterotype, which may promote a gut microbiome which is most closely taxonomically and functionally related to animals born to dams exclusively fed a control diet (hereafter referred to as "healthy"; Ma et al., 2014). We acknowledge that "healthy" and "dysbiotic" community states are not well defined in humans nor primates alike, and failure to revert to the control state may not, in fact, be dysbiotic but may represent an adaptive community. To begin to clarify this issue of adaptivity, we examined gut microbiome community structure as a true function of age and maternal diet.

Postnatal age structures the gut microbiome community

We next examined how enterotypes were distributed across time (age of offspring), with samples stratified by maternal dietary exposure. For juveniles with longitudinal sampling, we found all 6 month old juveniles on the maternal high fat diet transitioned to the high *Treponema* abundance enterotype at 13 months old (Fig. 6B). The 6-month-old juveniles on the maternal control diet had much more variable transitions, although a majority transitioned to the high *Treponema* abundance enterotype (Fig. 6A). All of the 3-year-olds, regardless of maternal diet, were observed to fall within the high *Treponema* abundance enterotype (Fig. 6). Interestingly, dams on the control diet were mainly observed to fall into both enterotypes, although animals with subsequent sampling ($n=3$) were observed to generally remain or transition to the high *Treponema* abundance enterotype (Fig. 6). In contrast, dams on the high fat diet with subsequent sampling ($n=2$) were primarily observed to occur or transition to the low *Treponema* abundance enterotype (Fig. 6). When we next examined if high fat diet exposure was associated with enterotype classification, we found the low *Treponema* abundance enterotype was relatively enriched in samples taken from animals exposed to a maternal high fat diet (Fisher's exact test, $p=0.06$), which appeared driven mainly by maternal diet (Fisher's exact test, $p=0.003$) as we found no significant difference in enterotype at 6 months, 1 or 3 years (Fisher's exact test, $p>0.05$); this time interval would have allowed for co-housing variation. When examining taxonomic differences by LEfSe, we found that animals only exposed to a maternal control diet had few differentially abundant taxa when compared to those exposed to a maternal high fat diet,

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even when weaned and maintained on a control diet for as great as 2.5 years (Supplemental Fig. 3). While we did not see taxa overlapping between control or high fat diet groups within the low *Treponema* cluster, we did find that *Ruminococcus* spp., *Fibrobacter, Anaeroplasma,* and *Lachnospiraceae* spp. were abundant in the high *Treponema* cluster regardless of diet. Furthermore, when examining co-occurrence when stratified by DMM cluster and diet, we saw that *Lachnospiraceae* had many significant positive co-occurrences ($p < 0.05$) while *Ruminococcus* had significant negative co-occurrences ($p < 0.05$).

Conclusions

We found that the gut microbiome of Japanese macaque offspring from 6 months to 3 years of age was structured by both maternal diet and post-natal age. We have previously published that maternal diet persistently effects the offspring microbiome in this highly relevant primate model of maternal high fat diet and obesity (Ma et al., 2014; Pace et al., 2018). Here we provide further evidence to demonstrate that the macaque gut microbiome is a representative model for interrogating the human microbiome throughout early life. This is in agreement with prior human data (Yatsunenko et al., 2012). Furthermore, alterations in alpha diversity by age within the gut microbiome are also in agreement with prior human studies (Yatsunenko et al., 2012), and may be due to decreases in *Bifidobacterium* that occur post-weaning. Specifically, we found a significant decrease in *Bifidobacterium* after 6 months, most likely due to the absence of mother's own breastmilk consumption.

In order to better model the gut communities beyond weaning, we employed DMM modeling and observed two main enterotypes characterized by high and low relative abundance of *Treponema.* In parallel with *Treponema* enrichment to high abundance, we observed co-enrichment of members of Clostridia, such as *Ruminococcus* and *Lachnospiraceae* ASVs. We find these findings to be intriguing since murine studies have demonstrated the effect of *Clostridia* on gut serotonin production and regulatory T lymphocyte induction (Atarashi et al., 2011; Gaboriau-Routhiau et al., 2009; Mathewson et al., 2016; Reigstad et al., 2014; Yano et al., 2015). Consistent with these observations were our findings (based on inferred metagenomics) that butanoate, a mediator of inflammation and modulator of gut serotonin production (Reigstad et al., 2014; Yano et al., 2015), temporally varied. To elucidate the mechanism of these interactions further, we examined the microbial interactions and ecology using co-occurrence modeling. We found that although *Treponema* per se did not have any significant detectable co-occurrences, positive co-occurrence events differed significantly between high and low *Treponema* groups ($p = 5.5^{e-8}$ by Fisher's Exact Test). These results suggest that *Treponema* may regulate the permissiveness of the overall commensal microbial community, most likely through an indirect mechanism to promote exclusion of detrimental microbes within a community. Altogether, the current study's findings are consistent with our prior observations and collectively suggest that high *Treponema* (seen with a maternal control but not high fat diet) may be a more restrictive enterotype which, in turn, promotes a "healthy" gut microbiome (Ma et al., 2014; Pace et al, 2018). We acknowledge that "healthy" and "dysbiotic" community states are not well defined in humans nor primates alike, and failure to revert to the control state may not, in fact, be dysbiotic but may represent an adaptive community as evidenced by its community ecology.

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With this in mind, we examined the ecologic mechanisms by which maternal dietary exposures influenced the high and low *Treponema* groups over time, even with switching the offspring to a control diet post-weaning for up to 2.5 years. We found that within offspring only exposed to a control diet (i.e., the dams were fed a control diet, and the offspring were weaned onto a control diet; control/control) we observed significantly greater variability in the transition between high and low *Treponema* enterotypes. This was particularly true in the 6 month to 13 month transition and between adults examined across multiple time points. This differed in high fat diet exposed animals, with all juveniles at 6 months transitioning to the high *Treponema* group by 13 months of age. Furthermore, adults consuming a high fat diet were more likely to remain in the low *Treponema* cluster. This is consistent with our prior data demonstrating that a high fat diet post-weaning diet was associated with a lower abundance of *Treponema* within the offspring gut microbiome (Ma et al., 2014). Finally, upon examining taxonomic differences stratified by DMM cluster and diet, we found that Clostridia (*Ruminococcus* and *Lachnospiraceae*) were differentially abundant in the high *Treponema* cluster regardless of diet. These findings reinforce the notion that in the macaque, *Treponema* may be regulating permissiveness of the commensal gut microbiome. Since the maternal diet significantly alters its relative abundance, the end result of the niche occupancy by *Treponema* is parlayed variation in the abundance of Clostridia taxa. Altogether, we found that the gut microbiome of the Japanese macaque is altered by age and maternal dietary exposure with a resultant alteration in *Treponema* and permissively associated Clostridia taxa (*Ruminococcus* and *Lachnospiraceae*).

Given our observations of maternal dietary influence, it is intriguing to postulate when this early life influence is occurring. We and others have previously demonstrated that we can employ metagenomics and 16S sequencing to detect and characterize a unique and low abundance, low biomass placental and neonatal microbiome (Aagaard et al., 2014; Amarasekara et al., 2015; Antony et al., 2015; Chu et al., 2016, Chu et al. 2017; Collado et al., 2016; Doyle et al., 2014; Jiménez et al., 2005; Leon et al., 2018; Prince et al., 2016; Satokari et al., 2009; Ardisonne et al., 2014; Bassols et al., 2016; Doyle et al., 2017; Gomez-Arango et al., 2017; Zheng et al., 2017; Rautava et al., 2012; Martinez et al., 2018; Borghi et al., 2018; Parnell et al., 2017). Furthermore, in an elegant set of experiments, Li et al (2019) recently demonstrated that memory $CD4^{+}$ T cells are generated during intrauterine development in the human fetal intestine, which are phenotypicially similar to innate-like lymphocytes previously described in mice that are dependent on microbes for their maintenance (Prince et al., 2014). While there are a few investigators who have questioned the ability to reliably distinguish a placental microbiota from contaminant controls (de Goffau et al., 2018; Lauder et al., 2016; Leiby et al., 2018; Theis et al., 2019), it is not the aim nor intent of the current study to describe the potential origins or source of the primate offspring gut microbiome. Suffice it to say, ongoing and future studies in humans and primates are necessary to further clarify the source and seeding of the offspring microbiome.

There are inherent strengths and weaknesses to our study. First, our study design enables us to parse the influence of the maternal versus post-weaning diet, and further deconvolute the impact of post-natal age. Second, we have modeled the ecology of the developing gut microbiome in the primate as a function of both maternal diet and developmental age. These have collectively led to a series of novel and significant observations.

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An evident potential weakness to our study is the small sample number. Fortunately, despite sample number limitations, by conducting a power analysis we determined we were able to robustly address our research questions. Specifically, the number of animals studied, number of samples sequenced, and depth of our sequencing enabled us to be adequately powered to reach statistical significance in our findings, as documented in our results and after controlling for multiple comparisons. Future studies related to the questions posed here may benefit from additional animals and further longitudinal sampling across a longer time interval. Additional limitations to our study include reliance on inferred metagenomics pathways, which is an acknowledged limitation with any 16S-based pathway analysis. Finally, since our animals were socially housed, and all animals in a single run shared the same diet, there is the potential for co-housing to confound our dietary analysis. However, given that there was a persistent impact on the offspring gut microbiome even when diet and co-housing was varied, the significant modifiers in our cohort were postnatal age and diet. Since juveniles exposed to both maternal control and high fat diets were co-housed when fed a post-weaning control diet, we conclude that co-housing alone was not the weighted effect modifier.

Nevertheless, based on the strength and significance of our findings reported herein, future examinations of the taxa and pathways involved using functional shotgun metagenomics to species and strain level resolution will enable determinations of how *Treponema* may be involved in regulating the commensal gut microbiome during crucial stages of development. Further, as we and the wider community of microbial ecologists and microbiome scientists continue to advance and refine culture-independent methodologies, we anticipate the inclusion of additional positive (e.g., mock communities) and negative controls in future studies will enable a more precise characterization of animal microbiomes. Knowing that in our animals our offspring are predisposed to metabolic disease as a result of maternal high-fat diet feeding, these and future studies are of great translational and public health importance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Research Highlights

- Maternal diet structures the postnatal gut microbiome in Japanese macaques.
- The Japanese macaque gut microbiome communities are distinguished by the relative abundance of *Treponema*, largely as a consequence of permissivity versus exclusivity of other taxa.
- Major transitions in the gut microbiome occur post-weaning likely due to cessation of nursing

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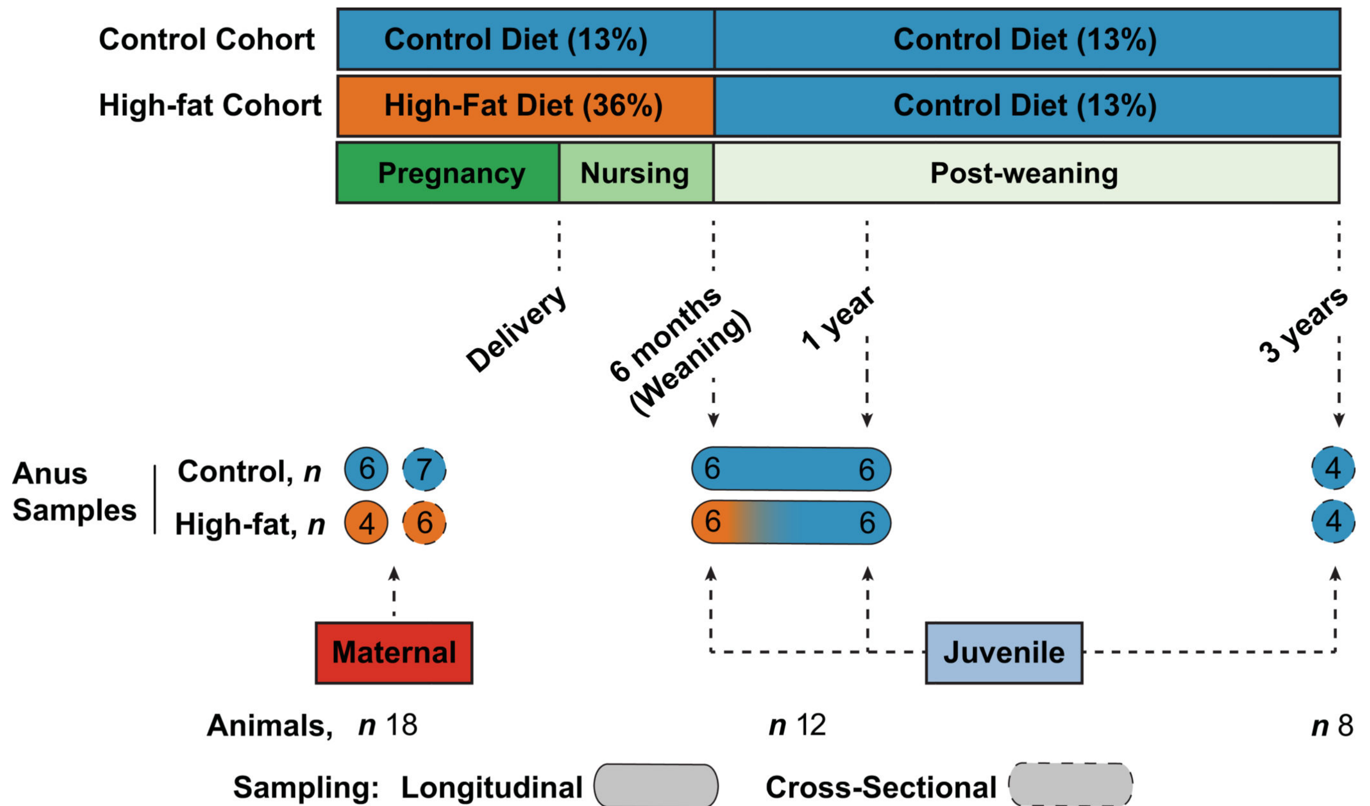

Figure 1. Study design.

Japanese macaque dams consumed either a control diet (13% fat) or high-fat diet (36% fat) during gestation and lactation. Offspring were maintained on the maternal diet until weaning, after which time the offspring were either kept on the control diet (control/control) or switched from their maternal high-fat to the control diet (high-fat/control). Maternal samples were collected during the third trimester glucose tolerance test (GTT). Offspring infant or juvenile samples were collected at 6 months, 13 months, or 36 months during scheduled metabolic testing or necropsy. The study design was primarily cross-sectional with longitudinal sampling between the 6- and 13-month time points as indicated.

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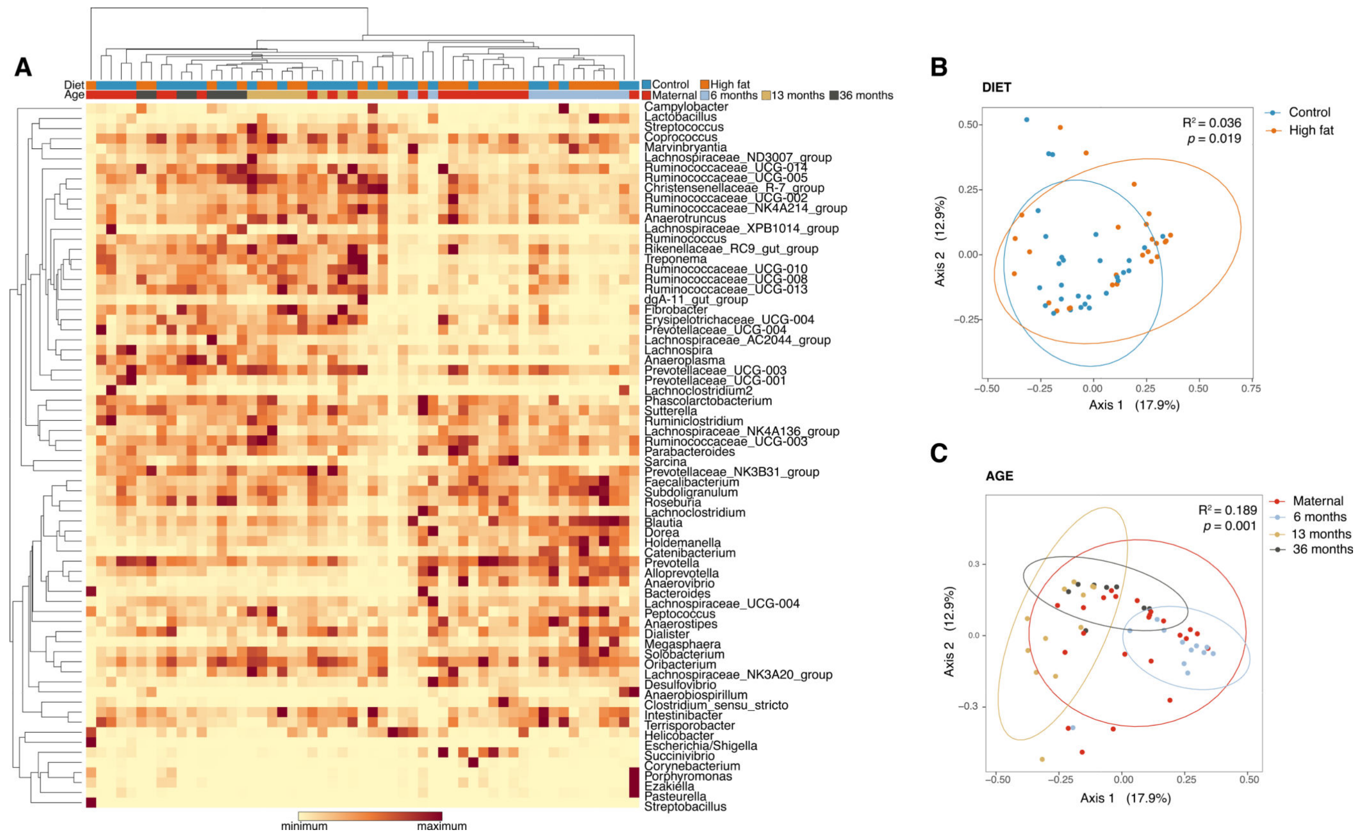

Figure 2. The Japanese macaque gut microbiome community membership and structure varies by post-natal age and maternal diet.

Anal swabs were collected from at various time points (6-month infant (*n*=12), 13-month juvenile (*n*=12), 36-month juvenile (*n*=8), and adult dams (*n*=23) during the third trimester of pregnancy) in our primate model of maternal high fat diet and obesity. (A) Hierarchal clustering of all taxa to the genus level using Spearman's correlation demonstrates that the gut microbiome are structured by age and diet. (B) Multidimensional scaling (MDS) demonstrates that beta diversity (Bray-Curtis) differs significantly when comparing high fat diet exposed animals to those only exposed to a control diet (p=0.019 by PERMANOVA, R^2=0.036). (C) MDS examination of beta diversity (Bray-Curtis) by age demonstrates significant alterations (p=0.0001 by PERMANOVA, R^2=0.189). When inputting the animal identification first into the PERMANOVA modeling, animal identification (with its nested diet) and age were retained after removing non-significant interaction terms (animal identification and nested diet: F model 1.29, R^2 0.67, p=0.006; animal age: F-model 7.386, R^2 0.104, p=0.001). Altogether, we find that the gut microbiome of the macaque is structured by age and maternal dietary exposures.

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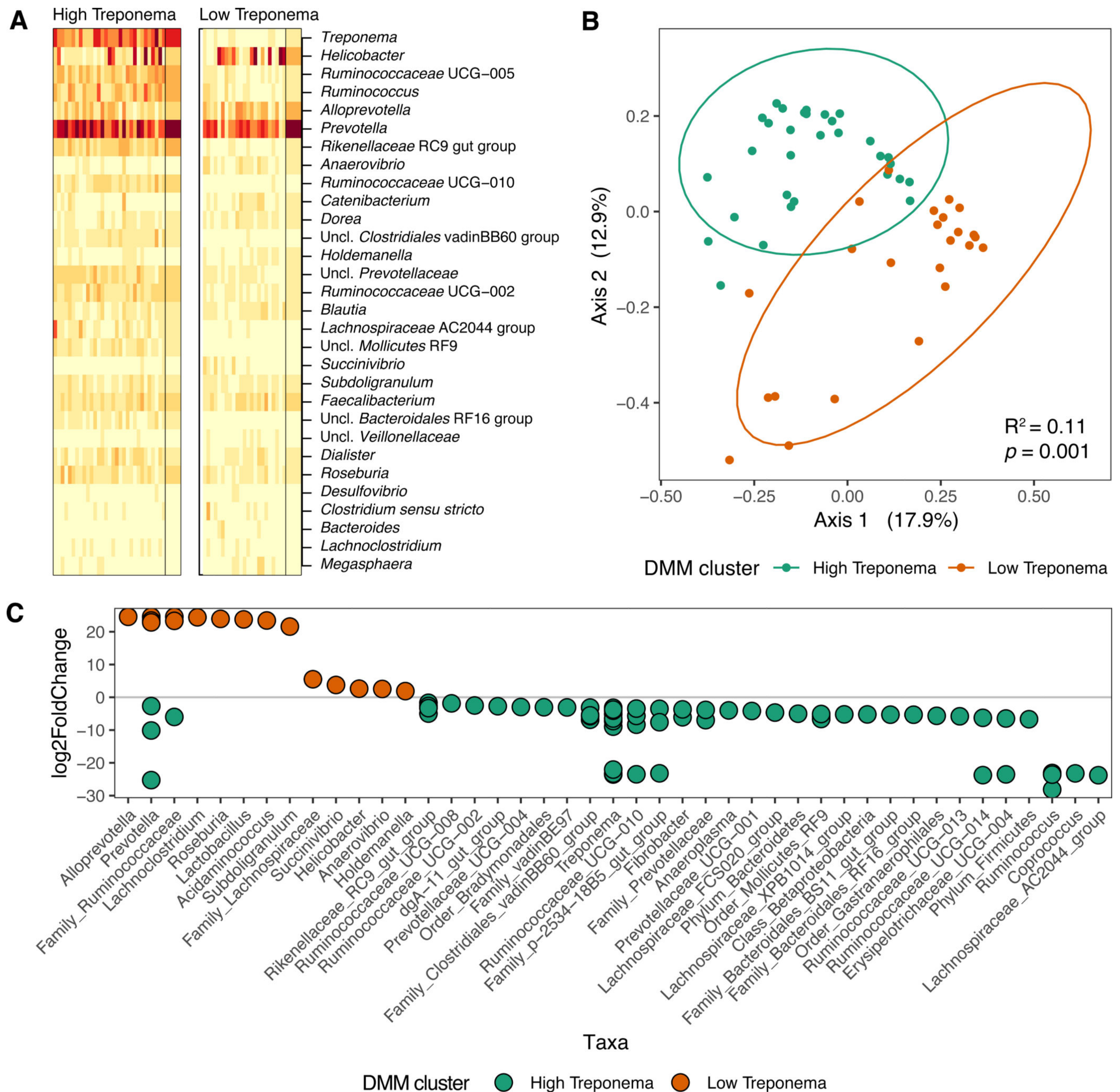

Figure 3. Dirichlet multinomial mixture modeling reveals distinct enterotypes that differ over time and with maternal diet.

(A) Heatmap of the top 30 genera. The first DMM component (left) is characterized primarily by a high abundance of *Treponema*. In contrast, the second DMM component (right) is characterized mainly by a low abundance of *Treponema*. Narrow columns represent samples and the two broader columns represent component averages. Cell colors represent square-root counts, with lighter and darker colors corresponding to smaller and larger counts, respectively. (B) Multidimensional scaling (MDS) ordination of Bray-Curtis distance

of samples demonstrates clustering based on DMM components (PERMANOVA, R^2=0.11, p=0.001). (C) Differentially abundant taxa identified via DESeq2.

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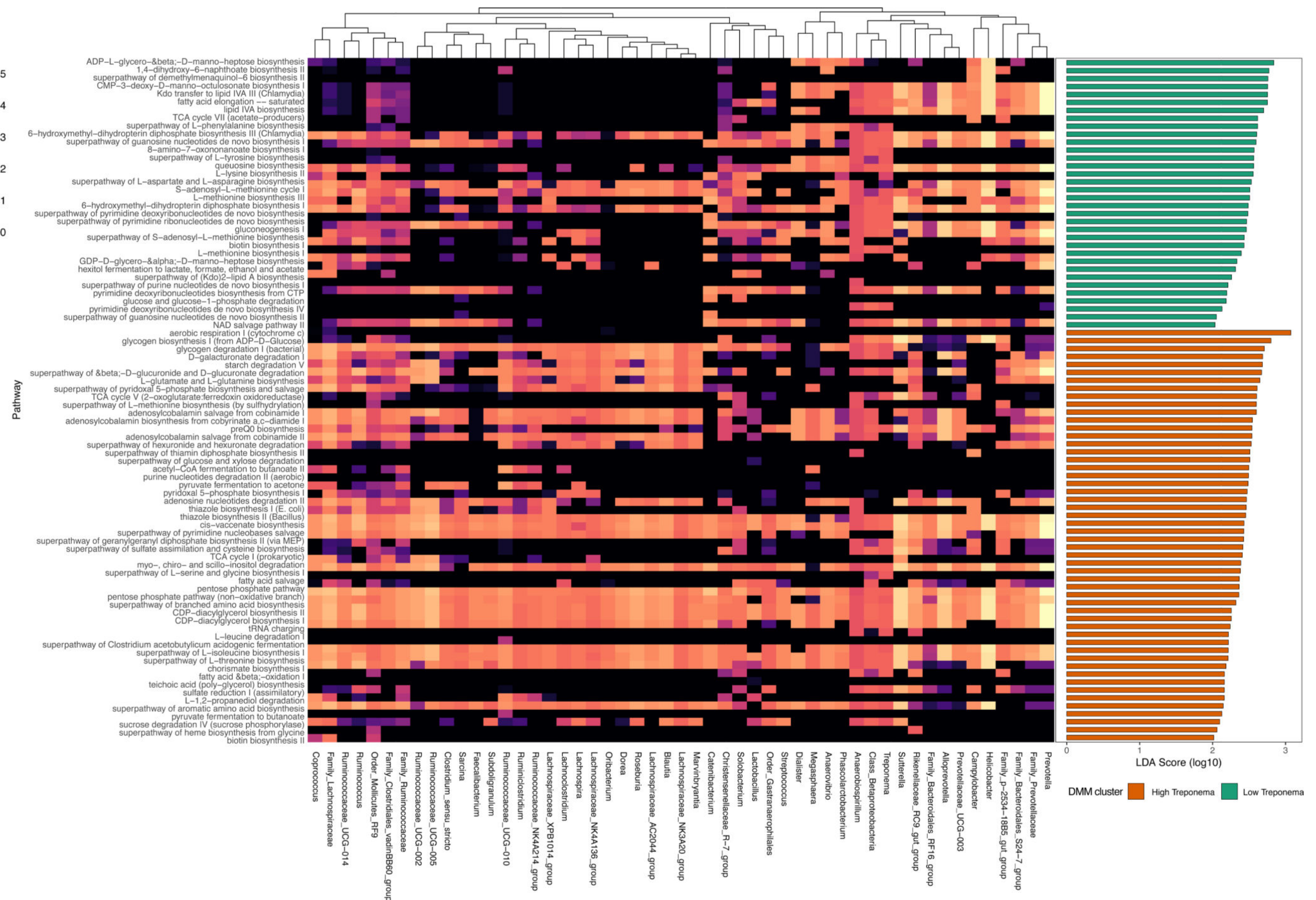

Figure 4. Inferred bacterial functional pathways abundance and enrichment.

Differentially enriched pathways based on DMM cluster were determined with LEfSe (right). Heatmap demonstrates the pathway abundance (log10 transformed) within the 51 taxa that were found to have the greatest contribution to the enriched pathways.

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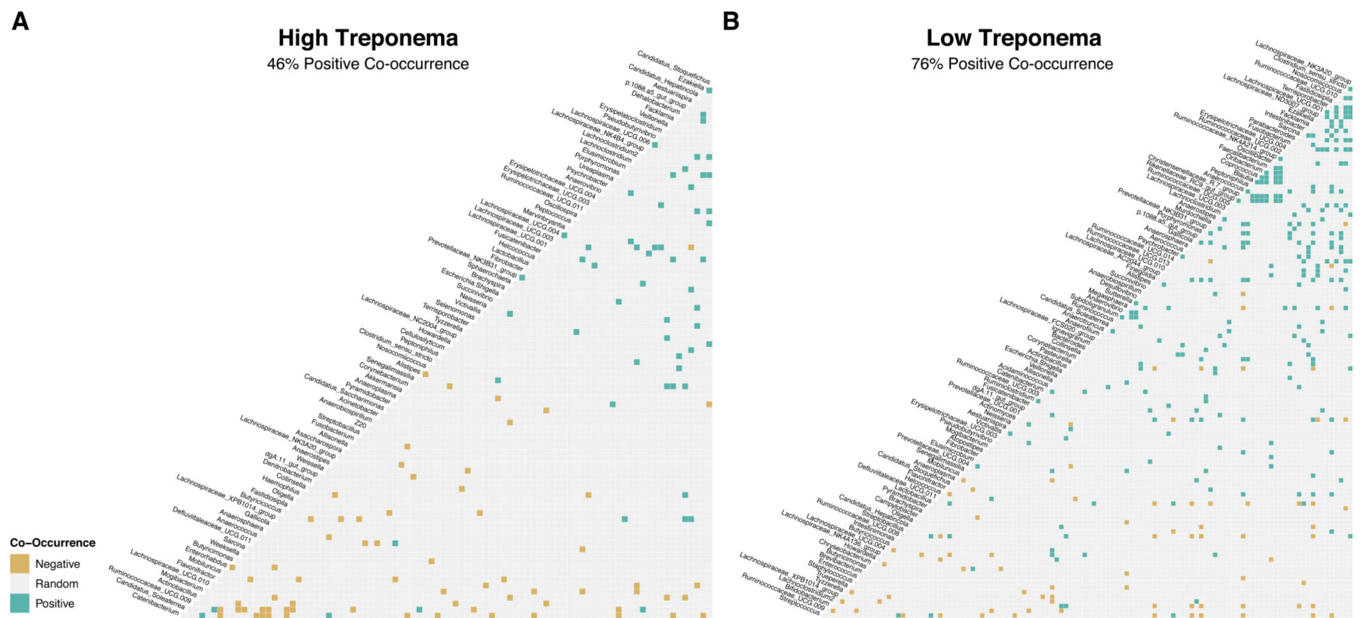

Figure 5. Probabilistic model of species co-occurrence suggests the relative low abundance or absence of *Treponema* promotes and ecologic environment which is permissive to microbial interactions.

Co-occurrence modeling of (A) high *Treponema* and (B) low *Treponema* clusters show that high *Treponema* clusters have fewer positive co-occurrence interactions (46%) when compared to low *Treponema* clusters (76%). This difference is significant (p=5.5^{e-8}) by Fisher's Exact Test with an odds ratio of 0.28. Negative co-occurrences are highlighted in yellow while positive co-occurrences are highlighted in green.

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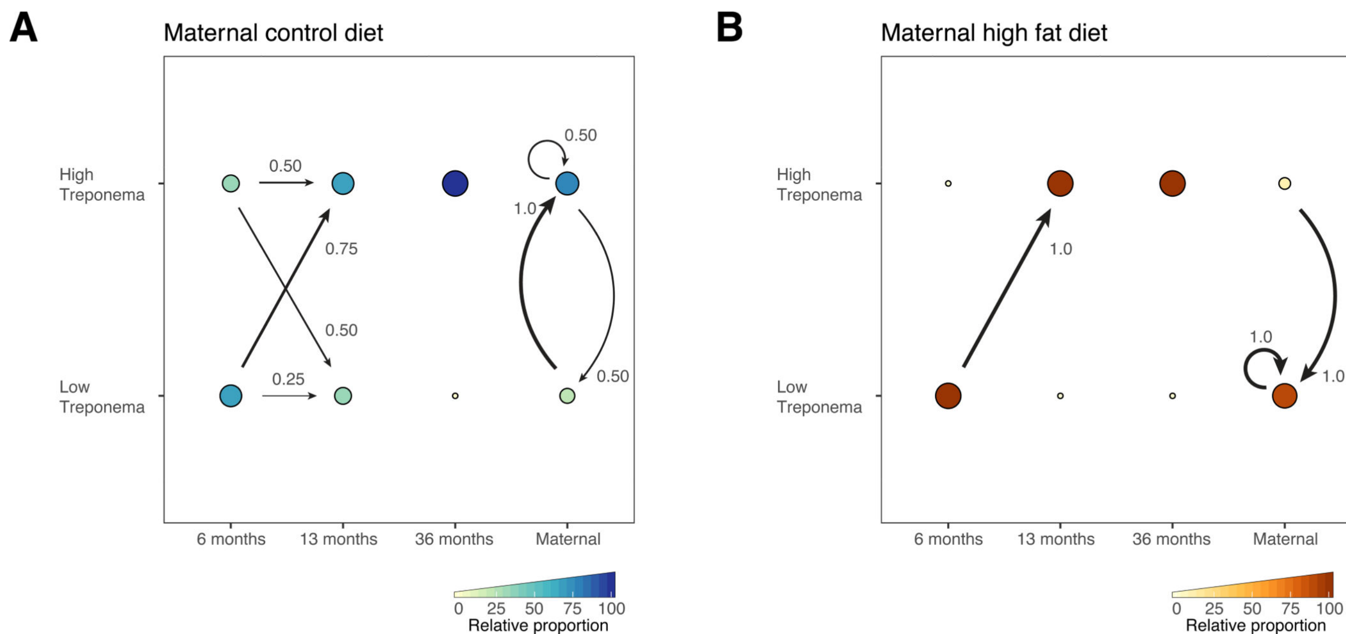

Figure 6. Community transitions in the gut microbiome of the Japanese macaque.

Proportions and transitions between and within DMM components over time stratified by maternal (A) control and (B) high fat dietary cohorts. Arrows indicate the transition probabilities in infant and juvenile offspring from 6 months to 13 months, and from subsequent samples taken from dams. Circle size and color, as detailed in the figure key, indicate the relative proportion of samples within each dietary cohort assigned to each DMM component. The very small circles indicate relative proportions of 0 (), except in the case of panel B where the small circle at 6 months indicates a relative proportion of 10%.

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Table 1.

Average (± standard deviation) read counts and Good's Coverage across maternal and offspring age strata and within diet cohorts, t test (Mann-Whitney).

		Read Count			Good's Coverage		
	n	**Control diet (*n* 29)**	**High-fat diet (*n* 26)**	***p***	**Control diet (*n* 29)**	**High-fat diet (*n* 26)**	***p***
Overall	55	36,728±820	36,428±878	0.80	0.9996±0.0002	0.9996±0.0002	0.88
Adults	23	36,074±1,272	34,338±1,417	0.37	0.9996±0.0002	0.9998±0.00009	0.15
6 months	12	36,893±1,836	36,061±1,074	0.70	0.9996±0.0002	0.9997±0.00004	>0.99
13 months	12	35,908±2,212	39,992±1,264	0.14	0.9996±0.0002	0.9994±0.0001	0.09
36 months	8	39,833±834	36,966±3,150	0.41	0.9996±0.0001	0.9995±0.0002	0.49