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Alterations in the Gut Microbiome at 6 Months of Age in Obese Latino Infants

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Abstract

Objectives: To investigate gut microbiome in Latino infants in relation to breastfeeding, obesity, and antibiotic exposure.

Methods—We analyzed the gut microbiome in 6 month old Latino infants from an on-going urban mother-child cohort. Alpha and beta diversity were assessed in relation to infants' early dietary exposure and anthropometrics including obesity.

Results: Infants exclusively breastfed at 4–6 weeks had lower alpha diversity and less bacterial abundance compared with those who did not. Breastfeeding status at 4–6 weeks and 6 months of age accounted for differences in alpha and beta diversity. Infants who were obese at 6 months of age had higher levels of alpha diversity compared with non-obese infants.

Conclusions: Early exclusive breastfeeding and obesity impacts microbial diversity by 6 months of age in Latino infants, a group at high risk for future obesity.

Keywords

microbiome; infant; obesity; latino; breastfeeding

Introduction

The microbiome is a complex ecosystem of microorganisms, including their related gene products and metabolites that colonize the gut [1]. The infant microbiome is established as the gut is colonized at birth and during the first few days, postnatally, by mode of delivery,

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Author Contributions:

AV analyzed data and authored manuscript, EL and BL provided assistance with manuscript, DZ conducted statistical analysis, and JW oversaw cohort selection, collected data, analyzed data, edited manuscript. All authors reviewed the final manuscript.

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early diet, and other environmental exposures, reaching a fully mature form by 3 years of age [2,3,4].

During infancy, the gut microbiome can be perturbed by diverse factors including diet, environmental exposures, and consumption of antibiotics.

Obese children have differences in gut microbiome compared to normal weight children; they have fewer protective bacteria, such as *Lactobacillus*, *Bifidobacteria*, and *Bacteriodes* [3,5]. Infants as young as 6 months of age have higher *Bifidobaterium* levels in association with normal weight status [6]. Obese children (6–16 years old) also have higher ratios of *Firmicutes* to *Bacteriocetes* and higher growth of the *Lactobacillus* ssp. [7]. Some this may be explained by breastfeeding. Breast milk colonizes the infant's gut with *Bifidobacterium* and *Lactobacillus* spp.. In contrast to breastfed infants, formula fed infants have higher levels of *Firmicutes*, which is associated with increased weight gain and future adiposity [7]. Early antibiotic use also alters the microbiome, resulting in decreased diversity [4,10]. The gut microbiome changes and associated risk of obesity confers increased risk for poor physical and mental health including risk of metabolic syndrome, cardiovascular disease, non-alcoholic fatty liver disease (NAFLD) and Type 2 Diabetes [11,12].

Our study compares alpha (within community) and beta (between community) diversity measures in fecal samples at 6 months of age. We hypothesized that exposures previously shown to be associated with obesity (e.g. no breastfeeding or early antibiotic exposure) would result in microbial changes.

Materials and Methods

The study participants are part of an ongoing, longitudinal urban, mother-child cohort entitled the Latino, Eating and Diabetes (LEAD) cohort (n=97). Details regarding recruitment, including inclusion and exclusion criteria are described in previous papers [13,14]. Briefly, mothers were recruited during pregnancy and children were followed from delivery, when anthropometrics (weight and length using standard digital scales) were assessed. At recruitment, maternal pre-pregnancy weight was collected based on self-report and height was measured using stadiometers. At birth, mode of delivery and infant birthweight were recorded. At 4–6 weeks of age and again at 6 months of age, child feeding practices—breastfeeding and introduction of any solids or non-breast milk liquids—were assessed. At 6 months of age, anthropometrics (weight and length) were again assessed and parents instructed on how to collect stool from diapers using a scoop attached to the lid of a sterile collection vessel prior to storage at 4°C [15]. A subset of the overall cohort (n=49) provided stool samples. By 6 months of age, almost all children are eating table foods regularly, and as such this is an important milestone age in infant development. Participants' stool samples were mailed to UCSF in an insulated mailer, overnight, while on ice packs. Stool samples were stored frozen at −80°C until they were batch processed by the Susan Lynch laboratory at UCSF.

Microbial Analysis

Assessment of the composition of the bacterial population was performed on extracted DNA from all retrieved stool samples using Illumina NextSeq (Illumina, Inc. Madison WI) sequencing of bacterial 16S rRNA gene amplicons at the Lynch laboratory at UCSF.

DNA was extracted using the QIAsymphony DSP Virus/Pathogen Mini Kit® (Qiagen GmbH, Hilden, Germany), with a previously described off-board bead-beating step [16]. DNA quantification was performed using the Qubit® 2.0 Fluorometer (InvitrogenTM, CA, USA) with the Qubit™ dsDNA HS Assay Kit (InvitrogenTM, CA, USA). PCR amplification reactions target the V4 hypervariable region of the 16S rRNA gene, as it is the most reliable regions representing full 16s rRNA [17]. Two PCR reactions were performed using previously published primers, 515F and 806R (Sigma-Aldrich®, MO, USA) [18]. Those samples that did not have successful amplicons were not added to the sequencing pool (n= 1/49). Sequencing reagents and the DNA library were prepared using the MiSeq Reagent Kit v3, 600 Cycles (Illumina, CA, USA) per manufacturer's instructions. Sample read numbers were representatively rarefied to 50,770 reads resulting in a rarefied operational taxonomic unit (OTU) table. Those samples had read numbers below 50,770 and were removed from analysis.

Ninety-eight percent (48/49) of those stool samples provided successful amplicons, and 94% (45/48) of the successful amplicons had quality filtered read numbers above the specified rarefied threshold and were included in downstream analyses

Statistical Analysis

Samples were assessed for microbial diversity. Specifically, alpha diversity was measured using the following diversity metrics: Shannon, Simpson, Inverse Simpson and Faith's Phylogenetic Diversity. Box plots were constructed summarizing richness (number of operational taxonomic units (OTUs) and evenness (how equal the abundances of the OTUs are). Kruskal-Wallis non-parametric tests to assess for statistical significance with $p<0.05$ defined as significant. Beta diversity) was measured using principal coordinates analysis using Bray-Curtis, Canberra and Weighted Unifrac and Unweighted Unifrac distance measures. Permutational multivariate analysis of variance (PERMANOVA) was used to compare groups and test the null hypothesis that the centroids and dispersion of groups as defined by space are equivalent. Significant PERMANOVA p-values indicate that microbial community differences are associated with stratification of the samples based on the indicated variable(s). Significant weighted and unweighted Unifrac findings indicate a strong phylogenetic component, where weighted UniFrac accounts for bacterial abundance and unweighted UniFrac considers only bacterial presence/absence. Bray-Curtis and Canberra metrics prioritize shared distributions of the most abundant or rare taxa, respectively, and do not take phylogenetic relatedness into account.

Potential predictors include feeding variables such as any breastfeeding and exclusive breastfeeding, defined as infant consumption of only human milk with no supplementation [19], at 4–6 weeks and 6 months of age or any introduction of 100% fruit juice or sugar sweetened beverages at 6 months of age. We also investigated delivery mode (vaginal versus

Cesearan delivery) and maternal pre-pregnancy body mass index (BMI) category (obese, overweight, normal/underweight) in relation to infant microbiome. Infant weight status includes obesity and being overweight at 6 month of age and body mass index Z score at 6 months of age, defined using the Center for Disease Control's growth curves with obesity defined as ≥95th percentile weight for length and overweight as ≥85th percentile weight for length [20].

Linear regression analysis was used to identify alpha diversity measures associated with these predictors, and PERMANOVA regression analyses for beta-diversity measures. Linear regression was also used to identify specific OTUs associated with feeding variables and weight status. Statistical significance was determined using Bonferroni 0.05/80/23=0.00002717391 for 80 OTUs and 23 phenotypes. For any predictors passing Bonferroni significance level, a stepwise forward selection procedure including all predictors were used to identify potential multi-factor effects. We further examined any breastfeeding at 6 months of age using our entire sample size with data on breastfeeding (n=45) and further assessed if there were any specific microbial changes related to introduction of non-breast milk liquids and foods in the context of breastfeeding by limiting the sample to only those who reported any breastfeeding (n=32). OTUs were excluded from regression analyses if they had over 50% of the samples with 0 counts.

Results

Breastfeeding and Juice Consumption

In infants who were exclusively breastfed at 4–6 weeks (n=22), there were higher levels of *Actinobacteria* (38.8% vs 24.1%) and lower levels of *Proteobacteria* (30.6% vs 39.6%). In those who exclusively breastfed at 6 months (n = 4), there were similarly higher levels of *Actinobacteria* (38.3% vs 13.9%) and lower levels of *Proteobacteria* (31.4% vs 44.5%). In infants who drank juice at 6 months, there were greater amounts of *Firmicutes* (27.9% vs 16.4%) and lower levels of *Actinobacteria* (28.4% vs 33.0%) and *Proteobacteria* (25.9% vs 39.0%). These findings were not statistically significant, however.

The exclusively breastfed infants at 4–6 weeks (n=22) and the infants receiving any breastmilk at 6 months (n=32) both had significantly less rich microbiomes (lower number of OTUs), (p = 0.035 and 0.009, respectively), and for those exclusively breastfeeding at 4–6 weeks had lower alpha diversity (Shannon's diversity, p = 0.046; Simpson, p=0.063 neared significance) (Figure 1).

Being breastfed at 6 months of age had the greatest impact on beta diversity in terms of consistently differentiating bacterial communities, explaining between 3.4% and 8.4% of the beta diversity (3.4% using the weighted UniFRac (p=0.002) and 8.4% using Canberra (p=0.006) metrics).

Any breastfeeding at 6 months (n=32) also consistently explained beta diversity between communities with 6% explained by the Bray-Curtis (p=0.006) and 8.5% by Canberra (p=0.006) metrics. Beta diversity, bacterial abundance and presence/absence metrics (weighted and unweighted UniFrac) between groups were also explained by exclusive

breastfeeding at 4–6 weeks (4% of diversity; p=0.014 and 2.7% of diversity p=0.033) and breastfeeding at 6 months (5.3%, p=0.001; and 3.4%, p=0.002).

Using the complete infant dataset (n=45), *Bifidocateriacae Bifidobaterium* (relative abundance 30%) differed among those who breastfed at 6 months of age (n=32) and those who did not with greater amounts among those who were breastfed (p=0.004; mean 0.37 versus 0.13). Restricting the sample to only infants whose mother reported any breastfeeding at 6 months, family and genus *Enterococcaceae Enterococcus* (1% abundance) ($p=1.44X10^{-5}$ for single variant association, p= 0.000344 after adjusting all other covariates) differed between those infants who were exclusively breastfed at 6 months (n=4) compared to those who were not, with higher amounts among those who were exclusively breastfed (mean 0.004 versus 0.08 in those non exclusively breastfed compared to those who were breastfed).

Obesity

In our cohort, none of the infants at birth were above 95 percentile in weight. The average birth weight for length z score was −0.40, and 6% of the infants' weight for length was above 95 percent (3/49). At 6 months, 16.7% of infants were overweight (n=9), with an average BMI z score of 0.4. At 1 year, 10.6% of infants were obese (n=6). At 1 year and 2 years, 12.2% of the infants were obese (n=6).

In stool samples from infants who were obese at 6 months (n=6), there were suggestive differences in presence of bacteria from the following phyla: higher levels of *Firmicutes* (36.9% vs 17.5%), and lower levels of *Actinobacteria* (24.8% vs 32.3%) and *Proteobacteria* (25.4% vs 36.7%), compared to normal weight infants (n = 39).

In those infants that were obese at 6 months as well as in those who were overweight at 1 year there was significantly more alpha diversity (Simpson's, p=0.025; Inverse Simpson's, p=0.25 for obesity and 6 months and Faith's, p=0.036 for overweight at 1 year) (Figure 1). Evenness and Shannon index neared statistical significance (Figure 2). BMI Z score at 6 months of age (p= 0.015) and weight for length Z score at birth (p=0.006) were most predictive in terms of shared distribution of the most abundant and rare taxa using the Bray-Curtis metric.

Similarly, being overweight at 6 months (n=13) (p=0.035), and higher weight for length Z score at birth (p=0.006) were able to differentiate abundant and rare taxa using Canberra. BMI Z score at 6 months (p=0.039), overweight at 6 months (p=0.046), and higher weight for length Z score at birth (p=0.028) were significantly able to predict bacterial abundance using the Weighted UniFrac metric. We did not find any relationship between maternal pre-pregnancy obesity status and alpha and beta diversity metrics in infants at 6 months of age. There was no association between sugar sweetened beverage consumption at 6 months or antibiotic exposures and alpha or beta diversity measures.

Discussion

Our study showed significant differences in microbiome of infants in our high-risk Latino cohort such as lower alpha diversity in exclusively breastfed infants at 4–6 weeks, lower abundance of *Bifidobaterium* in breastfed infants at 6 months of age, and higher *Enteroccoaceae Eneteroccucs* in those who exclusively breastfed 6 months of age.

Breastfeeding and Juice Consumption

We found lower levels of *Firmicutes* and an increased abundance of *Lactobacilli and Bifidobacteria* in Latino infants who received any breastmilk at 6 months. Previous studies have shown that the balance of lower *Firmicutes* and increased *Lactobacilli* and *Bifidobacteria* in breastfed infants correlates as a protective factor for risk of obesity [2,3,21]. Exclusively breastfed infants tend to have higher levels of beneficial phyla *Lactobacillus* [3] and lower levels of pathogenic *Clostridium difficile* [21]. This is consistent with previous findings for breastfed infants [22–25], as the breastmilk promotes oligosaccharides in the gut which promote growth of healthy *Bifidobacteria*, which is absent in formula [22,23].

Our infants who were exclusively breastfed at 4–6 weeks also had decreased alpha diversity similar to previous studies, and also had less richness as did those who received any breastmilk at 6 months of age. Breast-fed infants are consistently characterized as having decreased alpha diversity (less diverse colonization) and increased fractions of *Bifidobacteria* and *Lactobacillus*; in contrast, formula-fed infants have increased alpha diversity (more complex microbiota) with greater fractions of *Bacteroides, Clostridium,* and *Enterobacteria* [22–24].

Our results did not show significant changes in biodiversity in infants who consumed sugar sweetened beverages in contrast with previous studies [5]. Our cohort had a high percentage of obese and overweight pregnant mothers with potentially different microbial constitutions compared to those of lean mothers. Infant microbiome may have been altered by maternal diet although we did not directly assess maternal intake. Maternal diet can influence breastmilk composition including milk oligosaccharides, immunoglobulins and other macronutrients [26], 27]. High glucose diets lead to *Bifidobacteria* and decreased *Bacteroides* [28]. High sugar and high fat diets, Western diets, have also been shown to causing inflammation in the infant and thus long term effects irreversible through inflammation cause altered epigenetic programming such as methylation [29].

In our cohort, those who were exclusively breastfed at 6 months of age had significantly higher levels of *Enteroccocus*, although the sample size small (n=4). *Enteroccocus* has previously been found to be one of the early colonizers in breastfed infants to help prepare the gut for the strictly anaerobic *Bifidobacteria* which become main colonizers of the infant's gut later in infancy [23,30]. The role of *Enteroccocus* in early immune development is poorly understood, while other studies have noted that formula fed infants have persistently higher counts of *Enterococcus* in infancy, suggesting a role in more mature microbial constitution [31].

Ours is the first study to find higher numbers of *Enteroccocus* in exclusively breastfed infants compared to those infants who are mixed fed. It is possible that the as long as infants are maintained on breastmilk, they may continue to have a higher number of *Enteroccocus*, a more immature gut profile, compared to those who are exposed to formula in infancy.

Obesity and Antibiotic Exposure

Similar to other studies, we found higher levels of bacteria from the phylum *Firmicutes* [30] in obese patients, which is associated with increased energy absorption from the diet and mild inflammation [31], although our results were not statistically significant.

We found increased alpha diversity in obese 6 month infants in contrast with studies of adult cohorts that found lower alpha diversity (Shannon index) and less richness correlating with increased adiposity and obesity-related complications, such as cardiovascular disease [32,33]. It is possible that in young children exposed to early obesity, patterns may be reversed. In a study similar to ours of mother-child pairs, having a high percentage of maternal obesity (34%), young children had greater alpha diversity compared to those with lean parents even after adjustment for child's body mass index [34]. In our cohort, 33.3% of the mothers were obese, and 66.6% were overweight based on pre-pregnancy BMI. Meanwhile, we did not find any significant alpha and beta diversity differences related to maternal obesity status. Numerous studies suggest that the infant microbiome gains more diversity during the first year of life as new foods are introduced [35]. Although we also did not find any differences in diversity based on age of introduction of solids, it is possible that obese infants may have more advanced microbiomes for their age groups that allows for differential energy expenditure and adiposity gain based on exposures to maternal microbiome at delivery and breastfeeding.

Antibiotics and other Environmental Exposures

We did not find any significant decrease in diversity or abundance in the microbiome of infants with antibiotic exposure in contrast with previous studies possibly due to the small sample size of our population [2,36,37]. However, recent studies have suggested microbiome changes due to exposures ranging from antibiotic to chemicals contained in household disinfectant cleaners, with weekly use of disinfectants leading to increased odds of being overweight or obese as early as 3 years old, as well as associated changes in microbiome including increased abundance of *Lachnospiraceae* as early as 3–4 months of age [38, 39]. Future studies are needed to assess the relationship between environmental exposures and microbiome composition.

Conclusion

Ours is the first study to examine the microbiome of infants in an urban Latino cohort at high risk for future obesity finding significant differences in composition based on early exclusive breastfeeding habits and presence of obesity. Latino children have a high risk of obesity, higher than non-Latino Caucasians [40]. Our cohort, in particular, has a high prevalence of obesity with 12% of our cohort being obese by 2–3 years [41] Obesity risk at such an early age increases risk for chronic obesity and metabolic disease including

NAFLD, Type 2 Diabetes Mellitus among others. Our study indicates that by 6 months of age there may be important microbial differences leading to future obesity.

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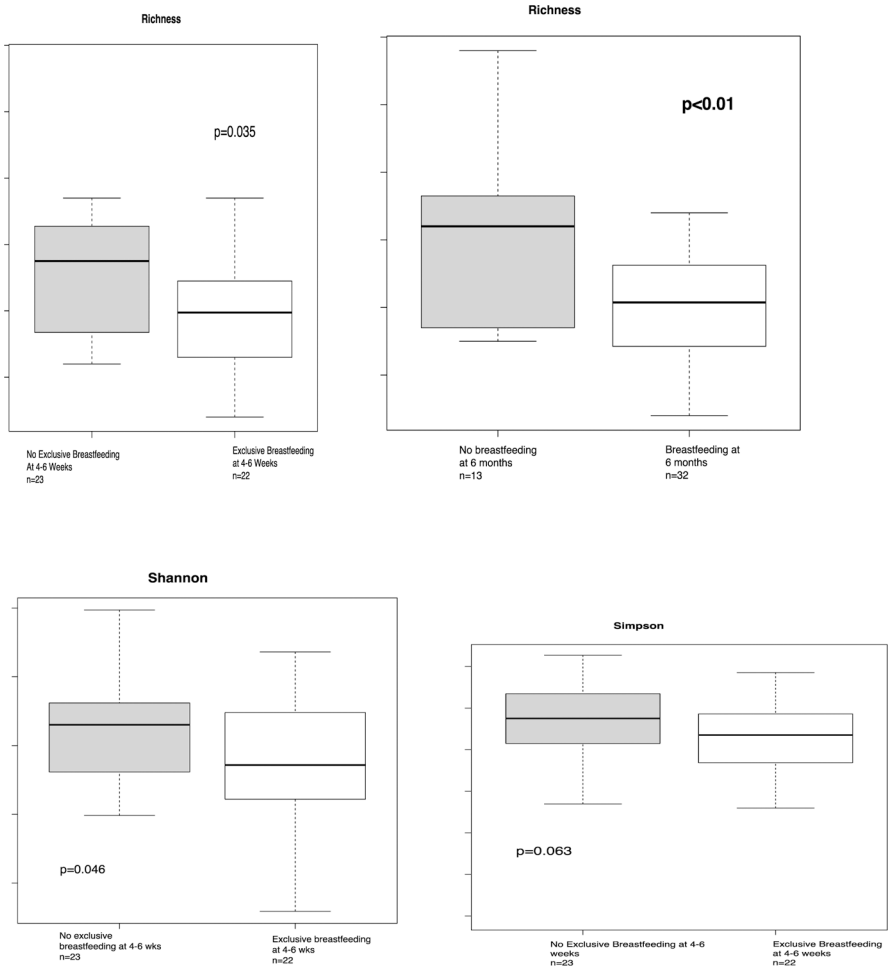

Figure 1:
Breastfeeding and Measures of Alpha Diversity

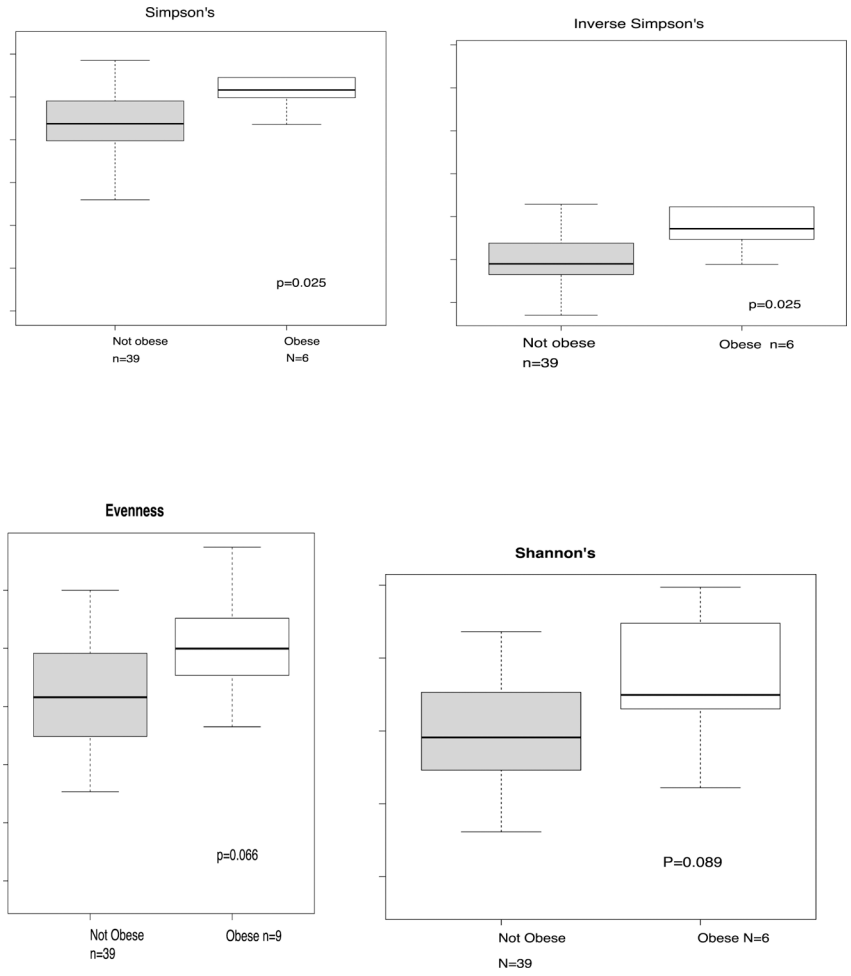

Figure 2:
Obesity at 6 months of age in Relation to Measures of Alpha Diversity