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## Gut Microbiota and Overweight in 3-year Old Children

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### Abstract

**Background:** The gut microbiota has been associated with overweight and obesity in adults, but the evidence in children is limited. Our aim was to study whether composition of the gut microbiota at the age of 3 years is associated with overweight/obesity in children cross-sectionally.

**Methods:** Children, who participated in a clinical trial of prenatal vitamin-D supplementation (VDAART), underwent standardized height and weight measurements, and collection of stool

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#### Contributions

Dr. Karvonen carried out the initial statistical analyses, interpreted data, drafted the initial manuscript, and reviewed and revised the manuscript; Dr. Sordillo designed sequencing assessment, interpreted data and reviewed and revised the manuscript; Drs. Gold, Bacharier, O'Connor, Zeiger and Beigelman acquired and interpreted data, revised manuscript critically for important intellectual content; Drs. Weiss, Litonjua and Gold designed the study concept. Drs. Weiss and Litonjua coordinated and supervised data collection, interpreted data, revised manuscript critically for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Supplemental material

Additional information on methods and results, as well as Tables E1-E9 and Figures E1 and E2 are found in the Supplemental material (Supplemental\_material.docx).

Supplementary information is available at International Journal of Obesity's website.

samples at three years of age. 16S rRNA sequencing (V4 region) of the stool samples were performed with Illumina MiSeq. Associations between microbiota and overweight/obesity (body mass index z-scores > 85<sup>th</sup> percentile) was analyzed using logistic regression.

**Results:** Out of 502 children, 146 (29%) were categorized as overweight/obese. Maternal pre-pregnancy BMI, birth weight and length, formula feeding during the first year, high frequency of fast food consumption, and time watching TV or computer screen at 3 years were the risk factors for overweight/obesity. Of the top 20 most abundant genera, high relative abundance of *Parabacteroidetes*(*Bacteroidetes*;*Bacteroidales*) (aOR(95%CI): 0.69(0.53, 0.90,  $p=0.007$ ) per interquartile increase) and unassigned genus within *Peptostreptococcae* family were inversely associated with overweight/obesity, whereas high relative abundance of *Dorea*(*Firmicutes*;*Clostridiales*) (1.23 (1.05,1.43,  $p=0.009$ )) was positively associated. Associations were independent of each other. No associations were found between diversity indices and overweight/obesity.

**Conclusions:** Our data suggest that some of the differences in gut composition of bacteria between obese and non-obese adults can already be observed in 3-year old children. Longitudinal studies will be needed to determine long-term effects.

## Introduction

Obesity is a worldwide public health concern and is a known risk factor for several diseases such as diabetes, cardiovascular diseases and cancer.<sup>1</sup> The prevalence of obesity in children and adults is increasing in both developed and developing countries. Overweight and obese children are prone to become obese adults, and obesity in childhood may be a risk factor for cardiovascular disease in adulthood due to increased blood pressure and decrease in high-density lipoprotein cholesterol (HDL) mediated by body mass index (BMI) in adulthood.<sup>2</sup>

Risk factors for childhood overweight/obesity reported in the literature include, among others: parental (especially maternal) obesity,<sup>3,4</sup> C-section delivery, formula feeding, antibiotic treatment (both in *utero* and after birth),<sup>5</sup> smoking during pregnancy and low maternal educational level.<sup>3</sup> High fat/carbohydrate diet, low physical activity, and time spent at the computer/TV are additional often correlated risk factors associated with child adiposity.<sup>6-8</sup> Many of these recognized adiposity risk factors have been hypothesized to influence the risk of overweight and obesity by directly or indirectly influencing the composition of the intestinal microbiota and the contribution of those microbiota to a pro-obesogenic breakdown and metabolism of foods.<sup>9</sup>

Previous studies have suggested that diversity of the gut microbiota is inversely associated with overweight/obesity.<sup>10</sup> *Bacteroidetes*/*Firmicutes* ratio has previously been associated with obesity; however, a recent meta-analysis of mostly adult studies did not find any association.<sup>11</sup> Other studies among adults have identified bacterial taxa that may be associated with overweight/obesity. The levels of *Bacteroidetes*<sup>12-15</sup> and its genera *Bacteroides*,<sup>15,16</sup> *Bacteroides-Prevotella* group,<sup>17,18</sup> and *Bifidobacterium*<sup>13,16,19-21</sup> as well as *Methanobrevibacter* (Archaea) have been shown to be decreased in gut microbiota of overweight or obese adults, whereas species in *Lactobacillus*<sup>12,13,15,17,20</sup> and *Roseburia*<sup>15,22</sup> have been enriched. Not all the results have been consistent.<sup>19,23</sup> Studies in children above 3

years of age have identified similar taxa, especially *Bacteroides* or *Bacteroides/Prevotella* group<sup>24–27</sup> that have been associated with overweight/obesity and in addition, have observed that obesity is associated with lower levels *Akkermansia muciniphila*.<sup>26,28</sup> Associations between *Faecalibacterium prausnitzii* in the gut and overweight/obesity have been inconsistent.<sup>24,26,27,29,30</sup>

We have recently shown that C-section birth was related to higher Shannon diversity index (which takes account both community richness [a simple count of the number of species or number of Operational Taxonomic Units, OTUs, in a sample] and evenness [how equal the abundances of the species or OTUs are]), higher levels of *Firmicutes* and *Proteobacteria* coabundance scores, and lower levels of *Bacteroidetes* scores; and breastfeeding with lower level of *Clostridiales* in the gut sample collected at the age of 3–6 months.<sup>31</sup> Due to the link between C-section and the risk of overweight/obesity,<sup>5</sup> these taxa might be related to the development of overweight and obesity.

Our overall objective was to determine whether gut microbiota among 3-year-old children are associated with overweight/obesity at the same age. In addition, we tested whether other covariates, that may predict overweight/obesity, mediates the observed association between bacterial genera and overweight/obesity in this cross sectional analysis at the age of 3 years.

## Methods

The study population consisted of children, who participated in the VDAART clinical trial (The Vitamin D Antenatal Asthma Reduction Trial), a two arm, double-blind, placebo controlled, randomized, clinical trial of vitamin D supplementation in the pregnant mother to prevent asthma and allergy in childhood in three clinical centers across the United States – Boston, MA, St. Louis, MO, and San Diego, CA.<sup>32</sup> Pregnant women, who either had a personal history of or whose partner had a history of allergies/asthma, were randomized (N=880) during the first trimester of pregnancy (10–18 weeks) to one of two treatment arms of a 4-year clinical trial: 4 000 IU Vitamin D + prenatal vitamins or 400 IU Vitamin D + prenatal vitamins. VDAART was approved by the Institutional Review Boards (IRB: 2009P000557) of the participating Clinical Centers and the Data Coordinating Center, with pregnant women signing informed consent at the first enrollment visit. A total of 502 children, who had stool samples and information on height and weight at the age of 3 years, were included into the present study.

Maternal pre-pregnancy BMI was ascertained by medical chart review. Questionnaires regarding baseline characteristics (e.g. maternal age, marital status, smoking during pregnancy, family income, and parental race/ethnicity) were administered at enrollment. Child's race/ethnicity was based on parental report. Information on child's gender, birth weight and height, and mode of delivery was abstracted from labor and delivery records.<sup>33</sup> Children were followed up with repeated quarterly questionnaires from the age of 3 months, including questions about breast- and formula feeding, antibiotic treatment, time spent watching TV or computer screen, sleeping time and time for active play separately on the weekdays and on weekends. Frequency of fast food consumption at the age of 3 years was asked using a question "How often has child eaten something from a fast food restaurant in

the last month?”. At yearly visits, weight and height were measured in a standardized fashion. Two people measured standing height using the Shorr Length Board®.

### **BMI based z-score**

Age- and sex -specific BMI z-scores and percentiles using national reference data were calculated using CDC SAS Macro (A SAS Program for the 2 000 CDC Growth Charts).<sup>34</sup> Children were classified as overweight/obese if their BMI z-score percentile was greater than the 85<sup>th</sup> percentile.

### **Diet in the past month in the 3-year follow-up**

In the 3-year questionnaire, parents were asked about children’s diet for the past month using total of 87 questions of intake frequency of different food items. Due to missing data, two diet scores were created by Principal component analyses (PCA) using VARIMAX rotation with 41 variables without missing data (N=502) and with total of 87 variables with missing data (N=378). In both PCAs, score 1 was related mostly with unhealthy food and desserts (e.g. white bread, fried chicken and brownies) whereas score 2 was related to healthy food (e.g. fruits and vegetables). PCA scores (1 and 2) of the 87 variables correlated highly with PCA scores (1 and 2) of 41 variables (N=378,  $r=0.92$  and  $0.81$ , respectively), and thus, PCA scores of the 41 variables were used in the further analyses.

### **Assessment of vitamin-D status**

Measurement of 25-hydroxyvitamin D [25(OH)D] has been described previously. Briefly, at the age of 3 years, circulating 25(OH)D was determined using DiaSorin Liaison® (see Supplemental Material).<sup>32,33</sup>

### **Stool sample collection and sequencing**

The methods of stool sample collection and sequencing are described in the Supplemental Material in detail. Briefly, the child’s stool sample was collected by parents and samples were stored in freezers until processing. Stool samples were not collected if the child received any oral antibiotics in the previous 7 days. DNA extraction was performed on stool samples, and the bacterial 16S rRNA sequencing (V4 region) was performed with the Illumina MiSeq. A total of 506 children’s stool samples were collected and out of those, 502 also had information on BMI (mean age 36.1 months; age range 33.2–41.1 months).

### **Relative abundance of taxa, diversity indices and functional pathways**

One read per operational taxonomic unit (OTU) were filtered from the OTU table. The average (SD) number of raw reads (>1read/OTU) across all samples was 51 713 (mean  $\pm$  SD = 51 713  $\pm$  21 756). The measurements of bacteria/archaea were based on relative abundance i.e. the percentage of reads of a taxon per the total reads in a sample. For adjusted models, the relative abundances of genera were log-transformed (natural logarithm) and divided by range between 25<sup>th</sup> and 75<sup>th</sup> percentiles per genus. Shannon diversity index (which takes account both community richness [a simple count of the number of species or number of OTUs in a sample] and evenness [how equal the abundances of the species or OTUs are]) and richness (total number of observed OTUs in a sample) were measured from

35 089 OTUs with the Vegan package in RStudio. In total, taxa from 13 phyla, 45 orders, 102 families and 244 genera were identified. Taxonomy is only the first step in considering how gut microbes may lead to child adiposity, and we are interested in the inferred function of the microbial communities, as this is another step towards understanding the biology underlying taxonomic associations. Thus, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to impute the functional metagenome (KEGG pathways) (see Supplemental Material).<sup>35</sup>

### Statistical analyses

Chi-square analysis was used for comparison between determinants and overweight/obesity to identify possible confounding factors. Mann-Whitney U test were used for comparisons between the relative abundances of phyla or top 20 most abundant genera among children who were overweight/obese and non-overweight/non-obese children and for comparisons between relative abundance of *Dorea* and time spent watching TV or computer screen, or consumption of fast food among children who were overweight/obese and non-overweight/non-obese children. Spearman's correlation coefficients were calculated. Logistic regression models (adjusted for maternal education level) were used to determine associations between an interquartile range increase in natural log-transformed bacterial abundance and odds of overweight/obesity at age 3. All the models were adjusted for maternal education level. Other potential confounders for the association between the gut microbiome and overweight/obesity were examined (all determinants with p-values <0.2 shown in Table 1), but they did not change the estimates, and so were not analyzed further. In addition, the models for diversity indices and overweight/obesity were adjusted for sampling age. The results are presented as adjusted odds ratios (aORs) and their 95% confidence intervals (95% CI). Two-sided  $p < 0.05$  was considered significant. In the sensitivity analysis, 21 (4%) underweight children (BMI z-score percentile was the 5<sup>th</sup> percentile or lower) were excluded from the reference (non-overweight/non-obesity) group. Bonferroni correction was used for multiple testing. The data were analyzed using SAS 9 (SAS Institute Inc., Cary, NC, USA).

## Results

### Risk factors for overweight/obesity

Out of 502 children, 146 (29%) children were defined as overweight or obese. Half of the children were African Americans and 32% were white. Several maternal and child determinants for child overweight/obesity were identified ( $p$ -values <0.2 in  $\chi^2$ -test, Table 1). After adjustment for maternal education level, maternal pre-pregnancy obesity, higher birth weight and length, formula feeding during the first year, high frequency of fast food consumption and time spent watching TV or computer screen at 3 years were the risk factors for overweight/obesity (Table 1). No associations were found between child overweight/obesity and gender, race, maternal age, marital status, mode of delivery, vitamin D level at birth (25(OH)D levels 30ng/mL vs. <30ng/mL) or at the age of 3 years, breastfeeding, antibiotic treatment until the age of 3 years (Table 1), the number of courses of antibiotics since birth (Supplemental material Table E1), smoking during pregnancy, family income, antibiotic treatment during delivery, preterm delivery (<37 weeks of the gestation), sleeping time during weekends or weekdays, and time for active play during weekends or weekdays

(data not shown). Time spent watching TV or computer screen during weekends tended to have similar effect as for weekdays, but it was weaker (data not shown). PCA factors 1 (data not shown) and 2 (Table 1) of diet did not have clear dose-response effect on overweight/obesity.

### Bacterial phyla, Firmicutes/Bacteroidetes ratio vs. overweight/obesity

At the phylum level, the most abundant phyla were *Firmicutes* (mean relative abundance 62.4%) and *Bacteroidetes* (24.2%), which made up the highest proportion of relative abundances of 13 phyla followed with lower levels of *Actinobacteria* (8.9%), *Proteobacteria* (2.2%) and *Verrucomicrobia* (2.1%). None of the relative abundances of the top abundant phyla were statistically different between children who were overweight/obese and non-overweight/non-obese children ( $p>0.2$ ), except *Verrucomicrobia*, which had the same association as *Akkermansia* genus (below), as it was the only genus within *Verrucomicrobia* that was detected (mean relative abundances: overweight/obesity 1.6% vs. non-overweight/non-obesity 2.3%,  $p$ -value 0.03). Tertiles of *Firmicutes/Bacteroidetes* ratio were not associated with overweight/obesity (aOR (95% CI) 1.41 (0.87, 2.27) for middle tertile and 1.08 (0.66, 1.76) for the highest tertile vs. the lowest tertile).

### Top 20 most abundant genera vs. overweight/obesity

At the genus level, the majority of the top 20 most abundant genera belonged to the *Firmicutes* phylum, and the genera were mainly within the *Clostridiales* order (Table 2). When the relative abundances of the top 20 genera were compared, the levels of *Ruminococcus*, *Akkermansia* and *Parabacteroidetes* were lower and the level of *Dorea* were higher (Mann–Whitney U test,  $p<0.05$ ) in children who were overweight/obese as compared to non-overweight/non-obese children (Figure 1). The majority of correlation coefficients within the top 20 abundant genera were between  $-0.3$  and  $0.3$  (see in the Supplemental material Table E2). The highest correlations were found between *Coprococcus* and an unassigned genus within *Lachnospiraceae* family (*U.* from now on) and *Clostridiales U.* ( $r=0.54$  and  $0.51$ , respectively).

Seven out of 20 genera were associated with overweight/obesity ( $p<0.1$ ) after adjusting for maternal education level (Table 3). High relative abundances of *Parabacteroidetes* (*Bacteroidetes; Bacteroidales*) and *Peptostreptococcae U.* (*Firmicutes; Clostridiales*) were inversely associated with overweight/obesity, and similar tendency were seen with *Ruminococcus* (*Firmicutes; Clostridiales*) and *Akkermansia* (*Verrucomicrobia; Verrucomicrobiales*) (Table 3). High relative abundance of *Dorea* (*Firmicutes; Clostridiales*) was positively associated with overweight/obesity and similar tendency were seen with relative abundances of *Streptococcus* (*Firmicutes; Lactobacillales*) and *Roseburia* (*Firmicutes; Clostridiales*). When multiple testing was taken account, none of the associations retained their significance ( $p$ -values for *Parabacteroidetes* 0.14 and for *Dorea* 0.17), except in sensitivity analyses when underweight children were excluded from the reference group (see below).

Within *Lachnospiraceae* family, *Dorea* was one of the 6 studied genera, which all tended to be risk factors for overweight/obesity (Table 3). However, the relative abundance of

*Lachnospiraceae* family was not significantly associated with overweight/obesity (aOR (95% CI) 1.22 (0.93, 1.60) per interquartile increase,  $p=0.15$ ). No significant changes were observed in the estimates, when the models were additionally adjusted for the frequency of fast food consumption (Table 4), or for race, maternal pre-pregnancy BMI or other known risk factors (listed in the Table 1) for overweight/obesity (data not shown), or when all 7 genera were mutually adjusted (Table 4).

### Diversity indices and overweight/obesity

The mean and standard deviation of Shannon diversity index and richness were  $5.01 \pm 0.47$  (range 3.24–5.92) and  $2\ 842 \pm 771$  (range, 66–7 133), respectively. No associations were found with Shannon index or richness and overweight/obesity (see the Supplement material, Table E3).

### Levels of relative abundance of *Dorea* and overweight/obesity

Relative abundance of *Dorea* was higher in children who were overweight/obese compared to children who were non-overweight/non-obese if the children watched TV or computer screen 1 h/day or more on weekdays (see Supplemental material, Figure E1). Relative abundance of *Dorea* was higher in children who were overweight/obese compared to children who were non-overweight/obese, if the children consumed fast food once per week (see Supplemental material, Figure E2). Associations between diet, antibiotic treatment and TV watching and high levels (>median) of *Dorea*, *Roseburia*, *Peptostreptococcae U.* and *Akkermansia* are reported in the supplemental material (Supplement material, Table E4).

### Effect modification on diet and time spent watching TV or computer screen

None of the associations between the relative abundances of seven genera and overweight/obesity were modified by breast- or formula feeding, the frequency of fast food consumption or time spent watching TV or computer screen (Supplemental material, Table E5) *i.e.* the estimates did not considerably change when the models were adjusted with the suggested effect modifiers.

### Functional pathways

G protein-coupled receptors, fatty acid elongation in mitochondria, fatty acid metabolism or biosynthesis, that have previously been linked to overweight/obesity,<sup>36,37</sup> were analyzed. The first two were rare in children (majority were not detected). None of the analyzed four pathways were associated with overweight/obesity, except fatty acid elongation in mitochondria, which tended to be inversely associated with overweight/obesity (Supplemental material, Table E6). Fatty acid elongation in mitochondria was not associated with the relative abundance of *Dorea*, *Parabacteroidetes* or *Peptostreptococcae U.* (data not shown).

### Sensitivity analyses

When underweights excluded from the reference group, the association between relative abundance of *Dorea* and overweight/obesity became stronger (aOR (95% CI) 1.31 (1.11,

1.55) per interquartile increase,  $p=0.0013$ ) and its significance remained when multiple testing was taken account ( $p=0.03$ ) (see the Supplemental material, Table E7).

### Race and sex

Ethnic and gender differences were tested using interaction term in the model. Analyses suggested that in some cases associations of relative abundances of *Parabacteroides*, *Dorea* and *Roseburia* with overweight/obesity were stronger for non-white children than for white children (Supplemental Table E8), and also stronger in boys than in girls (Supplemental Table E9).

### Discussion

Almost one third of the 502 3-year-old children that participated in this study were categorized as overweight/ obese. Maternal pre-pregnancy BMI, high birth weight and length, formula feeding during the first year, high frequency of fast food consumption, and time spent watching TV or computer screen at 3 years were the risk factors for overweight/ obesity. Of the top 20 most abundant enteral microbiome genera, high relative abundance of *Parabacteroidetes* and *Peptostreptococcae U.* were inversely associated with overweight/ obesity, whereas high relative abundance of *Dorea* was positively associated with overweight/obesity. Time spent watching TV or a computer screen on weekdays was positively associated with high levels of *Dorea* and similar tendency was seen with high frequency of fast food consumption. No associations were found with Shannon index or richness and overweight/obesity.

### Risk factors for overweight/obesity

We found similar risk factors for overweight/obesity that have been reported previously. The strongest risk factor with dose-response was the frequency of consumption of fast food, as was found recently in the cross-sectional study among 72,900 children from 17 countries.<sup>38</sup> Pre-pregnancy maternal obesity (BMI  $\geq 30$ ), high birth weight and height, and formula feeding were associated with the risk of overweight/obesity, as has been shown in previous studies.<sup>5</sup> In contrast to previous studies, C-section birth mode was not a significant risk factor for overweight/obesity in the present study or in the Danish birth cohort study.<sup>39</sup> Families should be guided to eat more healthy foods and minimize fast foods to attempt to prevent overweight/obesity in early childhood.

### Depleted genera in overweight/obesity

High proportions of *Parabacteroidetes* and *Peptostreptococcae U.* were inversely associated with overweight/obesity. In addition, we found a tendency that the relative abundances of *Ruminococcus* and *Akkermansia* were inversely associated with overweight/obesity. No other studies have linked *Peptostreptococcae U.* with lean children/adults. In line with our results, case-control studies in adults and older children (mostly Caucasians) have found similar inverse association between *Parabacteroidetes*,<sup>15,27</sup> *Ruminococcus*<sup>19</sup> and *Akkermansia*, and overweight/obesity.<sup>26,28</sup> In contrast to our results, in a case-control study among Japanese adults, *Ruminococcus bromii* and *obeum* were enriched in obese adults, where the non-obese comparison group was defined as borderline underweight (the mean



BMI 18.6kg/m<sup>2</sup>).<sup>40</sup> Comparison between studies may be difficult when the sample collection age, ethnicity of the study populations, methodologies for sample handling, extraction and sequencing technologies can vary between studies. The knowledge of the association between gut microbiota and obesity is limited, especially in children: most of the previous studies have used quantitative polymerase chain reactions (qPCRs), which quantify the targeted taxa, however, the method does not describe and quantify any other taxa that are not targeted a priori.

### Dorea and overweight/obesity

In line with our findings, higher level of *Dorea formicigenerans* was associated with overweight/obesity in adults.<sup>15</sup> Our NGS sequencing method was not able to go further than genus level. However, when *Dorea* with 5 other genera within *Lachnospiraceae* family, which tended to be associated with overweight/obesity, was analyzed, the relative abundance of *Lachnospiraceae* (at the family level) was not associated with overweight/obesity. In contrast, a recent birth cohort study from Canada (2/3 Caucasian mothers) with earlier collected stool samples (at the age of 3–4 months) showed that higher levels of relative abundance of *Lachnospiraceae* family was associated with overweight at the age of 12 months, but no association was found with samples collected at the age of 12 months.<sup>41</sup> A higher level of taxonomy may increase the background noise thus reducing any association. Other analytical methods, such as qPCR are needed to increase the accuracy of the taxa. Also, larger sample sizes are needed for studies in the future.

### Diet, antibiotic treatment and consumption of fast food and 7 genera

Diet and antibiotic treatment were not associated with seven genera (*Parabacteroidetes*, *Dorea*, *Roseburia*, *Ruminococcus*, *Peptostreptococcae U.*, *Streptococcus* and *Akkermansia*), even though they were associated directly with overweight/obesity. Interestingly in a recent early-life high-fat diet mouse model found that the diet decreased the relative abundances of *Bifidobacterium* and *Akkermansia*, and increased the level of *Dorea*.<sup>42</sup> In addition, none of the risk factors modified the effects between the relative abundances of the seven genera and the presence of overweight/obesity. One reason that no effect modification was observed might be that we restricted the analyses to only the most abundant 20 genera, which may not include the taxa that might have been modified by diet and antibiotic treatments. In addition, as have been seen with dietary intervention studies, the responses might be seen only at the species level.<sup>10</sup> Also, we had a cross-sectional data set: early childhood dietary determinants e.g. breastfeeding may not have strong effect on 3-year gut microbiota; even though they are risk factors for overweight/obesity at the same age.

### Gut microbiota

The gut microbiota changes with time, and it has been found to reach adulthood-like microbiota between ages 2–3 years. However, it has been suggested that the development of microbiota might undergo change for even longer.<sup>43</sup> Changes in the microbiota are most related to diet when infants start to ingest solid food. In the present study, the dominant phyla were *Firmicutes* followed by *Bacteroidetes*, as has been found also in adult studies.<sup>44</sup>

Obesity is associated with low-grade inflammation.<sup>45</sup> It has been suggested that the lipopolysaccharide (LPS), that is a part of the outer membrane structure in gram-negative bacteria, stimulates inflammation.<sup>46</sup> LPS is produced and released continuously in the gut, when membranes of cells of gram-negative bacteria are broken down. It has also been shown that there are structural differences in LPS generation by various stimuli<sup>47</sup> resulting in variation in immunological markers such as pro-inflammatory cytokines.<sup>45</sup>

In the U.S., both Type 2 diabetes mellitus and obesity have skyrocketed. Thus, future work on the relation of gut microbiota to childhood adiposity may have relevance for understanding the etiology of both obesity and Type 2 diabetes mellitus.<sup>48</sup>

The main strength of the present study is the objectively measured height and weight, as well as high resolution next generation microbiome sequencing data. Gut microbiome samples were collected in 3-year-old children. At that age, the gut microbiota is generally established and reflects an adult-like microbiome. A weakness of the study is the cross-sectional study design, in which causality of the associations cannot be verified. We cannot rule out that social desirability bias, which may affect subjective study measures such as dietary assessment, physical activity and screen time, could influence our study results. A general weakness of currently applied next generation amplicon sequencing methods is that the taxonomic resolution to the species level is hardly ever possible. However, the method described overall gut microbiota, and not just the prior targeted taxa that have been analyzed previously with qPCRs.

In conclusion, our data show that some of the differences in gut composition of bacteria between obese and non-obese adults can also be observed in 3-year old children. This suggests that changes in the gut microbiome that may predispose to adult obesity begin in early childhood. Further follow-up will determine whether these differences become more pronounced as the children get older and additional studies will be needed to identify possible mechanisms by which the gut microbiome composition predisposes to obesity.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations:

**25(OH)D**                      25-hydroxyvitamin D

<b>aOR</b>	Adjusted Odds Ratio
<b>BMI</b>	Body Mass Index
<b>OTU</b>	Operational Taxonomic Unit
<b>NGS</b>	Next Generation Sequencing
<b>VDAART</b>	Vitamin D Antenatal Asthma Reduction Trial

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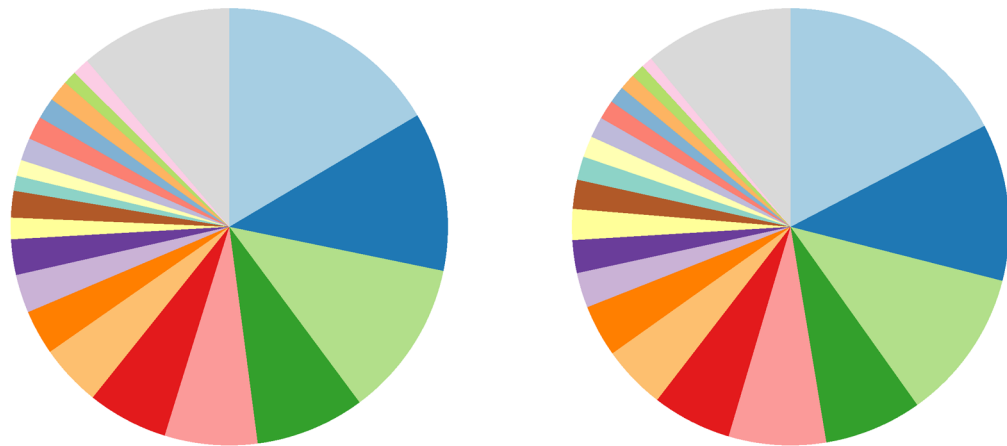
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Overweight/Obese

Non-overweight/Non-obese



<span style="color: #4682B4;">■</span> <i>Bacteroides</i> (16.5 vs. 17.4)	<span style="color: #FF8C00;">■</span> <i>Ruminococcus</i> (3.3 vs. 3.8)*	<span style="color: #9370DB;">■</span> <i>Coprococcus</i> (1.7 vs. 1.5)
<span style="color: #0000FF;">■</span> <i>Blautia</i> (11.8 vs. 11.6)	<span style="color: #D8BFD8;">■</span> <i>Prevotella</i> (2.8 vs. 2.6)	<span style="color: #FF6347;">■</span> <i>Roseburia</i> (1.7 vs. 1.4)
<span style="color: #90EE90;">■</span> <i>Lachnospiraceae U.</i> (11.6 vs. 11.2)	<span style="color: #483D8B;">■</span> <i>tRuminococcus</i> (2.6 vs. 2.4)	<span style="color: #4682B4;">■</span> <i>Streptococcus</i> (1.6 vs. 1.3)
<span style="color: #008000;">■</span> <i>Bifidobacterium</i> (8.1 vs. 7.2)	<span style="color: #FFFF00;">■</span> <i>Akkermansia</i> (1.6 vs. 2.3)*	<span style="color: #FFD700;">■</span> <i>Dorea</i> (1.6 vs. 1.2)*
<span style="color: #FF69B4;">■</span> <i>Faecalibacterium</i> (6.9 vs. 7.2)	<span style="color: #8B4513;">■</span> <i>Rikenellaceae U.</i> (2.0 vs. 2.2)	<span style="color: #90EE90;">■</span> <i>Erysipelotrichaceae U.</i> (1.0 vs. 1.0)
<span style="color: #FF0000;">■</span> <i>Ruminococcaceae U.</i> (6.0 vs. 5.9)	<span style="color: #ADD8E6;">■</span> <i>Parabacteroides</i> (1.1 vs. 1.7)*	<span style="color: #FFB6C1;">■</span> <i>Enterobacteriaceae U.</i> (1.3 vs. 0.8)
<span style="color: #FFA500;">■</span> <i>Clostridiales (order) U.</i> (4.6 vs. 4.7)	<span style="color: #FFFF00;">■</span> <i>Peptostreptococcaceae U.</i> (1.2 vs. 1.6)	<span style="color: #D3D3D3;">■</span> <i>Rest</i> (11.3 vs. 11.0)

**Figure 1.**

Mean relative abundances of the top 20 bacterial genera of A) overweight/obese and B) non-overweight/non-obese children. The rest of the genera have been summed up into a group 'Rest'. Percentage of the relative abundances in overweight/obese vs. non-overweight/non-obese children are in parentheses. \**p-value* <0.05 from Mann-Whitney-U test.

**Table 1.**

Determinants of overweight/obesity at the age of 3 years.

		Overweight/obesity				
		N	%	<i>p</i> -value <sup>1</sup>	cOR (95%CI)	<i>p</i> -value <sup>2</sup>
Maternal educational level	Less than high school	64	43.8		1	
	High school, Technical school	144	31.3		0.58 (0.32, 1.07)	0.08
	Some college	117	23.9		<b>0.40 (0.21, 0.78)</b>	<b>0.01</b>
	College graduate or Graduate school	177	25.4	<b>0.02</b>	<b>0.44 (0.24, 0.80)</b>	<b>0.01</b>
		N	%	<i>p</i> -value <sup>1</sup>	aOR (95%CI)	<i>p</i> -value <sup>2</sup>
Child sex	Female	233	26.2		1	
	Male	269	31.6	0.18	1.29 (0.87, 1.91)	0.21
Child race	White	163	27.0		1	
	Black, African American	251	30.3		0.95 (0.57, 1.58)	0.83
	Other	88	29.6	0.77	1.20 (0.67, 2.14)	0.55
Maternal age at enrollment	18-22	110	35.5		1	
	23-31	244	30.7		0.93 (0.55, 1.55)	
	32-39	148	21.6	<b>0.04</b>	0.60 (0.31, 1.15)	0.10
Maternal marital status	Not married	262	32.4		1	
	Divorced or separated	13	38.5		1.42 (0.44, 4.57)	0.56
	Married	227	24.7	0.13	0.78 (0.48, 1.28)	0.33
Maternal BMI pre pregnancy (missing, n=62)	Normal weight (18.5-24.9)	150	22.7		1	
	Underweight (<18.5)	13	7.7		0.28 (0.04, 2.27)	
	Overweight (25-29.99)	133	27.8		1.27 (0.73, 2.19)	
	Obesity (≥ 30)	144	38.9	<b>0.005</b>	<b>2.06 (1.21, 3.49)</b>	<b>0.007</b>
Mode of delivery	Vaginal	354	27.4		1	
	C-Section	148	33.1	0.20	1.27 (0.84, 1.93)	0.26
Birth weight	< 3 000 g	149	26.2		1	
	3000-3 500	189	26.5		1.03 (0.63, 1.69)	0.9
	> 3 500	164	34.8	0.15	<b>1.68 (1.02, 2.78)</b>	<b>0.04</b>
Birth length (missing, n=2)	< 49 cm	111	20.7		1	
	49-50	104	27.9		1.53 (0.81, 2.89)	0.19
	51-52.9	152	34.9		<b>2.16 (1.21, 3.85)</b>	<b>0.01</b>
	53-60	133	30.8	0.09	<b>1.93 (1.06, 3.53)</b>	<b>0.03</b>
	> 60	100	18.9		0.66 (0.36, 1.21)	0.19
Vitamin-D levels at birth (missing, n=99)	<30	147	34.7		1	
	≥ 30	256	25.4	<b>0.05</b>	0.66 (0.41, 1.06)	0.09
Reported breastfeeding in the first year of child's life (missing, n=3)	No	231	34.6		1	
	Yes	268	24.6	<b>0.01</b>	0.68 (0.44, 1.05)	0.08
Reported formula feeding in the first year of child's life (missing, n=3)	No	74	17.6		1	
	Yes	425	31.3	<b>0.02</b>	<b>1.94 (1.01, 3.71)</b>	<b>0.05</b>
Ab treatment until the age of 3 yrs (missing, n=8)	No	41	19.5		1	

				Overweight/obesity		
	Yes	453	29.4	0.18	1.60 (0.71, 3.57)	0.27
Frequency of fast food consumption in restaurant in the last month	Never or less than once per month	91	19.8		1	
	1-3 times per month	232	27.2		1.51 (0.83, 2.74)	
	Once per week	111	32.4		<b>2.00 (1.03, 3.88)</b>	<b>0.04</b>
	2-4 times per week or more	68	42.7	<b>0.01</b>	<b>2.83 (1.37, 5.85)</b>	<b>0.005</b>
Watching TV or a computer screen on the weekdays	One hour a day or less	107	20.6		1	
	1 to 3 hours a day	337	30.9		<b>1.72 (1.01, 2.91)</b>	<b>0.04</b>
	4-10 hours a day or more	58	34.5	0.08	1.88 (0.91, 3.89)	0.09
Vitamin D-levels at the age of 3 yrs (missing, n=53)	<30	381	29.9		1	
	30	68	20.6	0.12	0.69 (0.36, 1.31)	0.26
Diet: PCA factor 2, axis score (rich of vegetables, fruits)	1st quintile	101	40.6		1	
	2nd quintile	100	21.0		<b>0.39 (0.21, 0.74)</b>	<b>0.004</b>
	3rd quintile	101	26.7		0.57 (0.31, 1.04)	0.07
	4th quintile	100	24.0		<b>0.52 (0.28, 0.96)</b>	<b>0.04</b>
	5th quintile	100	33.0	<b>0.02</b>	0.81 (0.45, 1.47)	0.49

Overweight/obesity is defined as BMI z-score > 85 percentile at the age of 3 years. cOR Crude odds ratio. Adjusted models (aOR) are adjusted for maternal education level.  $p$ -value<sup>1</sup> for  $X^2$ -test,  $p$ -value<sup>2</sup> for logistic regression model.



**Table 2.**

Top 20 most abundant genera in 502 stool samples (in phylum, class, order and family order).

Taxonomy (phylum; class; order; family)	Genus	< DL	Mean	SD	Minimum	Maximum
Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae	<i>Bacteroides</i>	1	17.10%	12.96%	0%	61.92%
Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae	<i>Prevotella</i>	40	2.66%	7.34%	0%	65.44%
Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae	<i>Rikenellaceae U.</i>	15	2.10%	2.94%	0%	23.04%
Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae	<i>Parabacteroides</i>	17	1.55%	2.26%	0%	19.24%
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	<i>Dorea</i>	0	1.30%	1.44%	0.01%	15.88%
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	<i>Blautia</i>	0	11.66%	8.47%	0.02%	55.65%
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	<i>Coproccoccus</i>	1	1.57%	1.04%	0%	7.94%
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	<i>Roseburia</i>	2	1.51%	2.11%	0%	13.64%
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	<i>tRuminococcus</i>	0	2.49%	2.74%	0.11%	22.02%
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	<i>Lachnospiraceae U.</i>	0	11.31%	5.60%	0.22%	32.80%
Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	<i>Faecalibacterium</i>	0	7.10%	5.06%	0.01%	23.42%
Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	<i>Ruminococcaceae U.</i>	0	5.90%	3.24%	0.14%	19.26%
Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	<i>Ruminococcus</i>	2	3.69%	3.25%	0%	18.75%
Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae	<i>Peptostreptococcaceae U.</i>	2	1.46%	1.97%	0%	13.18%
Firmicutes; Clostridia; Clostridiales;	<i>Clostridiales (order) U.</i>	0	4.67%	2.94%	0.03%	20.45%
Firmicutes; Bacilli; Lactobacillales; Streptococcaceae	<i>Streptococcus</i>	1	1.34%	1.74%	0%	17.59%
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae	<i>Erysipelotrichaceae U.</i>	0	1.00%	1.27%	0.01%	11.99%
Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae	<i>Bifidobacterium</i>	0	7.47%	6.27%	0.01%	43.45%
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	<i>Enterobacteriaceae U.</i>	9	0.95%	2.73%	0%	26.93%
Verrucomicrobia; Verrucomicrobiae; Verrucomicrobiales; Verrucomicrobiaceae	<i>Akkermansia</i>	17	2.09%	3.59%	0%	28.16%

U. denotes "unassigned" at the genus level; &lt; DL the number of samples under detection limit; SD standard deviation

**Table 3.**

Unadjusted and adjusted associations between the top 20 most abundant genera in 502 stool samples and overweight/obesity at the age of 3 years (in phylum, order and family order).

Taxonomy (phylum; order; family)	Genus	Overweight/obesity			
		cOR (95%CI)	p-value	aOR (95%CI)	p-value
Bacteroidetes;					
Bacteroidales; Bacteroidaceae					
Bacteroidales; Bacteroidaceae	<i>Bacteroides</i>	0.90 (0.67, 1.21)	0.50	0.97 (0.72, 1.32)	0.86
Bacteroidales; Prevotellaceae	<i>Prevotella</i>	1.00 (1.00, 1.01)	0.75	1.00 (0.99, 1.01)	0.96
Bacteroidales; Rikenellaceae	<i>Rikenellaceae U.</i>	0.94 (0.78, 1.14)	0.54	0.93 (0.77, 1.13)	0.46
Bacteroidales; Porphyromonadaceae	<i>Parabacteroides</i>	<b>0.70 (0.54, 0.90)</b>	<b>0.006</b>	<b>0.69 (0.53, 0.91)</b>	<b>0.007</b>
Firmicutes;					
Clostridiales; Lachnospiraceae					
Clostridiales; Lachnospiraceae	<i>Dorea</i>	<b>1.21 (1.05, 1.41)</b>	<b>0.01</b>	<b>1.23 (1.05, 1.43)</b>	<b>0.009</b>
Clostridiales; Lachnospiraceae	<i>Blautia</i>	1.02 (0.79, 1.32)	0.86	1.02 (0.79, 1.32)	0.86
Clostridiales; Lachnospiraceae	<i>Coprococcus</i>	1.18 (0.94, 1.47)	0.16	1.15 (0.92, 1.44)	0.23
Clostridiales; Lachnospiraceae	<i>Roseburia</i>	1.11 (0.96, 1.28)	0.15	1.14 (0.98, 1.32)	0.08
Clostridiales; Lachnospiraceae	<i>tRuminococcus</i>	1.06 (0.91, 1.23)	0.43	1.06 (0.91, 1.24)	0.46
Clostridiales; Lachnospiraceae	<i>Lachnospiraceae U.</i>	1.10 (0.87, 1.41)	0.43	1.11 (0.86, 1.42)	0.43
Clostridiales; Ruminococcaceae	<i>Faecalibacterium</i>	0.90 (0.66, 1.22)	0.49	0.88 (0.64, 1.21)	0.43
Clostridiales; Ruminococcaceae	<i>Ruminococcaceae U.</i>	1.04 (0.79, 1.37)	0.79	0.99 (0.75, 1.31)	0.95
Clostridiales; Ruminococcaceae	<i>Ruminococcus</i>	0.80 (0.62, 1.03)	0.09	0.79 (0.61, 1.02)	0.07
Clostridiales; Peptostreptococcaceae	<i>Peptostreptococcaceae U.</i>	<b>0.84 (0.70, 1.00)</b>	<b>0.049</b>	<b>0.83 (0.69, 0.99)</b>	<b>0.037</b>
Clostridiales;	<i>Clostridiales (order) U.</i>	0.94 (0.72, 1.22)	0.63	0.92 (0.70, 1.20)	0.54
Lactobacillales; Streptococcaceae	<i>Streptococcus</i>	1.14 (0.99, 1.32)	0.07	1.14 (0.99, 1.32)	0.07
Erysipelotrichales; Erysipelotrichaceae	<i>Erysipelotrichaceae U.</i>	1.00 (0.86, 1.16)	0.99	1.00 (0.87, 1.17)	0.95
Actinobacteria;					
Bifidobacteriales; Bifidobacteriaceae	<i>Bifidobacterium</i>	1.17 (0.94, 1.46)	0.16	1.16 (0.92, 1.45)	0.20
Proteobacteria;					
Enterobacteriales; Enterobacteriaceae	<i>Enterobacteriaceae U.</i>	1.03 (0.99, 1.06)	0.14	1.03 (0.99, 1.06)	0.10
Verrucomicrobia;					
Verrucomicrobiales; Verrucomicrobiaceae	<i>Akkermansia</i>	<b>0.81 (0.67, 1.00)</b>	<b>0.046</b>	0.84 (0.68, 1.03)	0.09

U. denotes "unassigned" at the genus level; Overweight/ obesity is defined as BMI z-score >85 percentile; cOR Unadjusted Odds Ratio; aOR Adjusted Odds Ratio; 95%CI 95% Confidence limits; Models are adjusted for maternal education level and are expressed as change in interquartile range (ln-transformed).

**Table 4.**

Adjusted associations between relative abundances of 7 genera and overweight/obesity at the age of 3 years.

		Overweight/obesity					
		Model 1		Model 2		Mutually adjusted	
Taxonomy (phylum)	Genus	aOR (95%CI)	<i>p</i> -value	aOR (95%CI)	<i>p</i> -value	aOR (95%CI)	<i>p</i> -value
Bacteroidetes;	<i>Parabacteroides</i>	<b>0.69 (0.53, 0.91)</b>	<b>0.007</b>	<b>0.68 (0.52, 0.90)</b>	<b>0.006</b>	<b>0.72 (0.54, 0.94)</b>	<b>0.018</b>
Firmicutes;	<i>Dorea</i>	<b>1.23 (1.05, 1.43)</b>	<b>0.009</b>	<b>1.21 (1.04, 1.41)</b>	<b>0.016</b>	<b>1.23 (1.04, 1.45)</b>	<b>0.015</b>
Firmicutes;	<i>Roseburia</i>	1.14 (0.98, 1.32)	0.08	1.15 (0.99, 1.34)	0.07	<b>1.18 (1.01, 1.39)</b>	<b>0.04</b>
Firmicutes;	<i>Ruminococcus</i>	0.79 (0.61, 1.02)	0.07	0.81 (0.62, 1.04)	0.10	0.86 (0.66, 1.13)	0.28
Firmicutes;	<i>Peptostreptococcaceae U.</i>	<b>0.83 (0.69, 0.99)</b>	<b>0.04</b>	<b>0.82 (0.68, 0.98)</b>	<b>0.03</b>	<b>0.75 (0.61, 0.91)</b>	<b>0.005</b>
Firmicutes;	<i>Streptococcus</i>	1.14 (0.99, 1.32)	0.07	1.15 (0.99, 1.33)	0.06	<b>1.17 (1.00, 1.37)</b>	<b>0.05</b>
Verrucomicrobia;	<i>Akkermansia</i>	0.84 (0.68, 1.03)	0.09	0.84 (0.68, 1.04)	0.11	0.88 (0.72, 1.08)	0.22

U. denotes "unassigned" at the genus level; Overweight/obesity is defined as BMI z-score >85 percentile. aOR Adjusted Odds ratio; 95%CI 95% Confidence interval; Estimates are expressed as change in interquartile range (ln-transformed). Model 1 is adjusted for maternal education level; Model 2 is additionally adjusted for the frequency of fast food consumption. In mutually adjusted model, all the 7 genera are adjusted simultaneously and with maternal education level.