

**The effect of Vitamin A Supplementation,
Vitamin A status and other immunity
features on the microbiome of infants**

M.Sc. Thesis

**Submitted to the Robert H. Smith Faculty of Agriculture, Food
and Environment,
The Hebrew University of Jerusalem, School of Nutritional
Sciences**

For the degree of "Master in Nutritional science"

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**Rehovot, Israel
December 2018**

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Acknowledgments

First, I wish to thank my supervisor, Prof. Ram Reifen, for the support, encouragement and professionalism which allowed me to fulfill my potential and explore new fields.

Second, I would like to thank Dr. Omry Koren and his lab members: Oren Ziv, Hadar Neuman and Nir Werbner for their specialism, patience and warm hospitality.

Third, I would like to thank my lab members: Dr. Shimrit Bar-El Dadon and Zipi Berkovich for all their assistance, advice and companionship.

Last, I would also like to thank Dr. Eitan Winter for his professional help in bioinformatics.

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Abstract

Introduction

Adequate (vitamin A) VA level is required for normal organogenesis, proper growth, immune competence, tissue differentiation, proper gastrointestinal activity, and vision.

VA is considered a comprehensive immunomodulator. In first-line defense, Vitamin A deficiency (VAD) impacts the integrity of gut mucosal barrier and the secretion of IgA, whereas in the adaptive response, VAD adversely affects the generation of T cells, especially Th17, iTreg and FoxP3⁺Treg.

VAD affects about 250 million preschool children, mainly in developing countries, where it increases morbidity and death from severe infections. High-potency VASUP is intended to boost liver stores, enabling the gradual release and delivery of VA to tissues in children with dietary VA deficits.

Microbiome is another entity associated with immune aspects. The impact of the microbiome is on both innate and adaptive phases of the immune system, i.e. it interacts with T cells, B cells and gut epithelial immune cells.

The gut microbiome in infancy is influenced by mode of delivery, gestational age, type of feeding, ethnicity, weight gain and other factors. The intestinal microbiota of term infants is dominated by 4 major phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*.

VA can modulate immune responses directly, by communicating with immune cells, or indirectly, by interacting with the microbiome. First, VAD can lead to dysbiosis by influencing the relative proportion of some gut bacteria. Second, VA signaling in B cells is essential for normal microbiome composition. Third, synergy between VA and microbial signals promotes Th17 differentiation. Last, VAD adversely affects the fucosylation process, which is essential for the support commensal microbiota. Lately, a connection between certain probiotics and improvement of VA status also has been investigated.

Microbiome, in turn, can affect VA metabolism. The stimulation of Dendritic Cells through bacterial signals increases the production of VA. On the one hand, *Bifidobacterium infantis*, which is sampled by immune cells, results in increased expression of VA metabolism enzymes, and on the other hand, the anti-inflammatory effect of *B. infantis* also depends on the presence of VA.

We hypothesize that VASUP, which improves the immunological status, will also dramatically change the microbiome composition.

Methods

A community based comparative study was conducted in Uganda. 100 Pairs of preschool children (6 – 24 months of age) and their mothers were recruited for the study. Among the included children, there were VAD and VAS children.

A time-series data collection started at baseline, i.e. before receiving VASUP, and continued at three more time points: a day after VASUP, a week after VASUP and a month after VASUP.

Out of 100 children that have been recruited, 67 children have completed full collection, and those were further analyzed.

Questionnaires regarding anthropometric, immunological and nutritional data were collected from child-mother dyads.

Blood samples for VA evaluation were collected twice and analyzed via iCheck FLUORO in field and via HPLC in lab.

Stool samples for the microbiome (16S rRNA) analysis were collected four times and the analysis included: DNA isolation, PCR and purification of its products, quantification and pooling of the samples to perform Illumina MiSeq sequencing.

16S rRNA Gene Sequence Analysis was performed in various bioinformatics and biostatistical tools: QIIME, LefSe, MaAsLin, PICRUSt.

Results

The infant microbiome population of our study consisted of 4 major phylas: *Actinobacteria* (48.6%), *Firmicutes* (27.1%), *Proteobacteria* (16.3%) and *Bacteroidetes* (7.4%). *Bifidobacterium* Genus constituted almost all of *Actinobacteria* Phylum (47%). Another dominant bacteria were *Clostridiales* order (18%), *Enterobacteriaceae* family (15.7%), *Veillonela* genus (13.7%), *Lactobacillales* order (9.1%) *Streptococcus* genus (6.2%) and *Prevotellaece* family (4%).

When we examined the individual within distances, we found there has been a significant difference in the distances of the microbial community between before

VASUP and a day after VASUP (FDR, $p=0.0009$), and also, between before VASUP and a week after VASUP (FDR, $p=0.008$). In addition, we discovered significance when comparing distances of a day after VASUP and a month after VASUP (FDR, $p=0.02$). No differences in specific bacteria were found due to VASUP.

Independently of VASUP, specific bacteria significantly differed by VA status of the children at baseline: *Betaproteobacteria* class, *Clostridiales* order, *Lachnospiraceae* family, *Lactobacillales* order.

The major metadata characteristic to differ in the microbiome composition was PCV. We discovered both Alpha and Beta diversity differences between the group of children who received PCV and those who did not. We found 21 bacteria that were more represented at the PCV recipients group.

Pre-natal growth, post-natal growth and gestational malaria also affected specific bacteria of children microbiome: *Coriobacteriaceae* and *Blautia*; *Leuconostoc* and *Lachnospiraceae*; and *Bacteroides*, respectively.

Importantly, we tested correlations between VA and growth, immunity and nutritional aspects of our metadata. We discovered that proper VA status is associated with better post-natal growth. We found a connection between antibiotics usage and also PCV reception and a higher VA level of children. We revealed a connection between maternal diet and VA status.

As a side analysis, we discovered a set of metadata within interactions regarding growth, immunity of children and maternal health and nutrition.

Discussion

Our findings on the general study microbiome match the current research of infant microbiome, with certain bacteria being a distinguished part of it.

We witnessed subtle influence of VASUP on the composition of the microbiome at the individual level. Previous studies also described the effect of small variation among populations compared to variation within populations. The microbial change within a week, and surely within a day, should be referred to the supplementation effect. We revealed other metadata characteristics, which had an impact on the microbial community, so it was challenging to conclude on the sole effect of VASUP on the microbiome. Furthermore, we believe that exclusive breastfeeding, a major factor that influences infant microbiome, could have masked the impact of VASUP on the microbiome.

Interestingly, we discovered that VA status of infants at baseline influences the composition of the microbiome with some bacteria of interest that were more abundant at the VAS group. We know of a connection between certain bacteria we found, such as members of *Lactobacillales*, *Clostridiales*, *Lachnospiraceae*, and VA.

Streptococcus pneumoniae is a predominant cause of pneumonia and it is a leading killer of children under 5 years of age, mainly in developing countries. PCV protects against half of serotypes causing the disease in those countries. There are studies that have already analyzed the nasopharyngeal microbial shift in PCV-vaccinated infants. Here we show an innovative connection between PCV and gut microbiome. We discovered significant differences in phylogenetic between and within distances, and specific bacteria of interest that were more represented in the PCV group: *Barnesiellaceae*, *Bacteroides*, *Lactobacillus agilis* and *Blautia producta*.

Moreover, we found correlation between infant growth, course of gestation to a specific bacteria which are relevant in the literature to those features.

To reinforce previous findings and add to them, we showed VA status relates to post-natal growth, child antibiotics usage, PCV reception and maternal nutrition.

Conclusion

Overall, we presented a complex set of correlations referring to infant's VASUP, VA status, growth and immune state, as well as mother's course of gestation, And the effect of these on the microbiome of the infant.

We found that VASUP slightly affects the microbiome of the recipients. The effect is mostly at the individual level. We could not spot specific bacteria which change as a result of VASUP.

Interestingly, we revealed distinctions in the microbiome composition of children with different baseline VA status.

Moreover, we discovered metadata growth and immune feature such as: PCV, pre-natal and post-natal growth, gestational malaria, that influence the microbiome in various extents.

There is a major concern among physicians and nutritionists as to whether nutritional supplementation alters the composition of the microbiome. Here we have shown that high dosage VASUP has an effect, yet a subtle one, on the microbiome.

1 Abbreviations

Vitamin A (VA)

Vitamin A Deficiency/Deficient (VAD)

Vitamin A sufficient (VAS)

Vitamin A Supplementation (VASUP)

Birth Weight (BW)

Low Birth Weight (LBW)

Pneumococcal Conjugate Vaccine (PCV)

Operational Taxonomic Units (OTU)

Quantitative Insights Into Microbial Ecology (QIIME)

Linear discriminant analysis effect size (LEfSe)

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)

Multivariate Association with Linear Models (MaAsLin)

Time Point (TP)

Segmented Filamentous Bacteria (SFB)

2 INTRODUCTION

Vitamin A (VA) and Vitamin A Deficiency (VAD)

Adequate VA level is required for normal organogenesis, proper growth, immune competence, tissue differentiation, proper gastrointestinal activity, and vision¹.

VA is considered a comprehensive immunomodulator. In first-line defense, VAD impacts the integrity of gut mucosal barrier and the secretion of IgA, whereas in the adaptive response, VAD adversely affects the generation of T cells, especially Th17, iTreg and FoxP3⁺Treg^{2,3}.

The vast majority of VA in the body is stored as retinyl esters in the liver. Peripheral tissue requirements are met by hepatic release of VA with its carrier, retinol-binding

protein, complexed with transthyretin. Across a wide range of adequate VA intake, serum concentrations of retinol remain stable, i.e. homeostatically controlled. However, nutrient-poor diets and recurrent infections, common among young children in developing countries, may lead to depletion of VA reserves in the liver and peripheral tissues. As the concentration of VA falls below 0.70 mmol/gram of liver, there appears to be a parallel decline in serum retinol concentration^{4,5}.

Generally the Cut off criteria for VAD is a blood VA level that is lower than 200 µg/L (or 0.7 mmol/l).

Vitamin A deficiency (VAD) affects about 250 million preschool children, mainly in developing countries, where it increases morbidity and death from severe infections^{6,7}.

Vitamin A Supplementation (VASUP)

High-potency VASUP is intended to boost liver stores, enabling the gradual release and delivery of VA to tissues in children with dietary VA deficits.

The public health efficacy of VASUP is well established. More than two decades of research have illustrated health benefits and reductions in mortality, nutritional blindness and anemia^{4,6}.

In Uganda, infants receive VASUP of 100,000 IU at 6 months of age, as a routine.

Microbiome in infancy

Microbiome is another entity associated with immune aspects. The impact of the microbiome is on both innate and adaptive phases of the immune system. The microbiome involves with T cells such as Th17 cells and FOXP3. It also affects B cells, gut epithelial cells and lymph nodes⁸.

Exposure to variety of appropriate bacteria in infancy is crucial for immunological development. It has been suggested that the infant gut is colonized by microorganisms before, during and after delivery^{9,10}. Although a stable microbiome may not be established until 1 to 3 years old, the infant gut microbiota appears to be an important predictor of health outcomes in later life¹⁰. As it presented in literature, the gut microbiome in infancy, is influenced by mode of delivery, gestational age, type of feeding, ethnicity, weight gain and other factors⁹⁻¹¹. The intestinal microbiota of term infants is dominated by 4 major phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*¹¹.

Connection between VA, the Microbiome and the Immune System

VA can modulate immune responses directly, by communicating with immune cells, or indirectly, by interacting with the microbiome, which in turn, can affect VA metabolism.

VAD diets increases the total amount of bacteria in the gut and alters the intestinal microflora of mice by decreasing the relative proportion of *Lactobacillus spp.* and simultaneously increasing the appearance of *Escherichia coli* strains¹².

It has been found that VAD increased bacterial translocation, which influences the severity of disturbances of the intestinal epithelium. This can lead to dysbiosis, a condition that can disrupt the normal process of immune-cell development, intestinal tolerance, and homeostasis¹³.

A study regarding the immune system showed that retinoic acid signaling in B cells is essential for oral immunization and microbiome composition¹⁴.

Synergy between retinoic acid and microbial-driven signals promotes Th17 cell differentiation². VAD Diet in mice elicited high levels of MUC2 by goblet cell hyperplasia and subsequently reduced the gut microbiome, including segmented filamentous bacteria (SFA), which drives the intestinal Th17 responses in mice. This suggests that RA deficiency can alter the gut microbiome, which, in turn, inhibits Th17 differentiation¹.

Moreover, VAD can alter the structure of fucosylated glycoproteins secreted by goblet cells¹⁵, by decreasing the production of ILC3s and cytokines¹³. This may adversely affect the fucosylation process, which is essential for the support commensal microbiota.

The microbiota plays a role in the biosynthesis and metabolism of vitamin A. The stimulation of DC through TLR2 increases the expression of host genes associated with the production of retinoic acid. Specialized DC subsets located in intestinal epithelial cells are responsible for producing retinoic acid from VA, resulting in the conversion of naive T cells into Foxp3C Treg cells¹⁶.

It has been documented that *Bifidobacterium infantis*, which has anti-inflammatory activity, is sampled by mucosal DCs, resulting in increased expression of RALDH (retinaldehyde dehydrogenase, an enzyme that produces retinoic acid from retinal). Indeed, we can witness a synergic interaction as the anti-inflammatory effect of *B. infantis* also depends on the presence of retinoic acid¹⁷.

A connection between certain probiotics and improvement of VA status also has been investigated. It has been demonstrated *in vivo* that probiotic bacteria could be used to deliver vitamin A to the tissues of a mammalian host¹⁸. In addition, other study proposed to engineer a probiotic bacterium that will produce b-carotene in the intestine, which will be metabolized to vitamin A¹⁹.

Study Hypothesis

We hypothesize that VASUP, which improves the immunological status, will also dramatically change the microbiome composition.

Study Objectives

First, we intended to assess whether VASUP at the age of 6 months old given to Ugandan infants has an impact on their microbiome at 3 time points post supplementation.

Second, we intended to assess if there is a difference in the microbiome of VAD and VAS infants at baseline.

Third, we were interested to investigate correlations of the vast metadata we collected with the microbiome, including immune aspects of children, maternal health and nutrition.

Last, we wanted to look into interactions of VA and other metadata in our study, as well as interactions within our metadata.

Ethical aspect of the study

The study has been approved by "The Mbale Regional Hospital Research and Ethics Committee (MRHREC)", The Republic of Uganda (registration number: UG-REC-012). "Collaborative Institutional Training Initiative (CITI)", a training course for Investigators and staff involved primarily in Social or Behavioral Research with human subjects, was completed by the researchers prior to the beginning of the study. The participant mothers signed an informed consent for them and for their children before participation.

3 MATERIALS AND METHODS

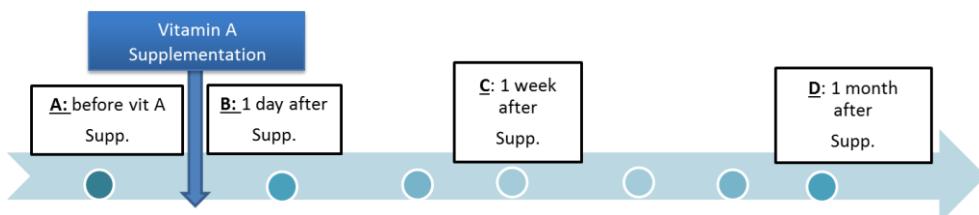
3.1 Study Design

A community based comparative study was conducted at Kumi health Centre IV in Kumi district, Uganda. The prevalence of VAD children (6-60 months of age) in Uganda is around 26-37%²⁰. 100 Pairs of preschool children (6 – 24 months of age) and their mothers were recruited for the study (Fig 2). Among the included children, there were VAD and VAS children. The children were exclusively breastfed. We excluded critically ill children, those with disabilities to take anthropometric measurements. We also excluded children if the children or the children's mothers used antibiotics less than one month before sample collection.

The national VASUP program was launched in 2002 in Uganda²¹. Since then, more and more children are being routinely supplemented with VA in the form of retinyl palmitate at the age of 6 months and then 12 months. In this study the follow up is around the first VASUP. The VASUP is given as drops to the mouth. The dosage is 100,000 I.U (30mg), a mega dose compared to the RDA (500 µg/day).

A time-series data collection started at baseline, i.e. before receiving VASUP (TP:A), and continued at three more time points (TPs): a day (TP:B) after VASUP, a week (TP:C) after VASUP and a month (TP:D) after VASUP.

Figure 1: Study timeline of data collection



Out of 100 children that have been recruited, 67 children (268 samples) have completed full collection (at all 4 times), and those were further analyzed (Fig 2).

The Health Centre team has received guidance regarding collecting and treating the samples, documenting the data collection and using necessary equipment. The nursing officer in Kumi hospital, the study coordinator was in charge of the collection throughout the study.

3.1.1 Assessment of Health, nutrition and demography

At baseline, the participant mothers who came to the Health Centre were required to fill in a structured questionnaire which included information on anthropometry and growth of the children, pregnancy and delivery characteristics of the mothers, as well as health and nutrition features of the children and mothers (Table 1).

3.1.2 Anthropometric data collection

Children were taken Anthropometric measurements including age, height and weight and were classified by percentiles of weight for age, weight for height and height for age according to WHO standards²² and Fenton Charts for premature infants²³. Children's Growth patterns were characterized by criteria for growth retardation²⁴.

3.1.3 Blood sample collection and analysis

About 3 ml of venous blood was collected at baseline and a month after VASUP. VA levels in child's blood were first tested in Kumi Health Centre using iCheck™ FLUORO (BioAnalyt, Teltow, Germany), special field measurement equipment which employs innovative technology of measuring the auto fluorescence of VA in whole blood samples. The analysis was performed as described in company's protocols; <http://www.icheckacademy.org/measurements.aspx>. As shown before, determination of blood VA levels by fluorometry method is a viable choice for field studies of vitamin A status^{25,26}. For keeping the samples safe in Uganda and outside of it, they were stored at -20°C, after serum separation procedure using a DSC-200A-1 centrifuge (Digisystem, New Taipei City, Taiwan) at 2500 rpm for 10 minutes. For reassurance of the results, we analysed serum vitamin A levels via High-performance liquid chromatography (HPLC) System as previously described²⁷: First, Ethanol and Hexane (JT Baker, Deventer, The Netherlands) were used for extraction in the Sample analysis. Second, Retinol Acetate R4632 and Retinol R7632 (Sigma-Aldrich, Missouri, USA) were used as standards, the former was used as an internal standard. Third, Methanol and 1% Acetic Acid (JT Baker, Deventer, The Netherlands) functioned as the mobile phase. The HPLC system consisted of Multi wavelength detector MD-910 (JASCO, Tokyo, Japan) (set at the wave length of 330nm, which is unique for VA), Auto sampler AS-95010 (JASCO, Tokyo, Japan), Pump LC-1150 (GBC, Braeside, Australia) and Column RP-C18 LiChroCart 125-4 (Merck, Darmstadt, Germany). Last, The data was analysed by Borwin-PDA ,version 1.2 (JMBS, France) software and provided us with the amount of retinol in each sample.

3.1.4 Stool sample collection

About 3 grams of stool sample were collected from the children at all 4 TPs (mentioned earlier) and were stored at -20°C in Uganda and outside of it. The stool samples were examined for gut microbiota using 16S rRNA sequencing.

3.2 Stool Sample Analysis

3.2.1 16S rRNA Polymerase Chain Reaction (PCR) Amplification and Sequencing

Microbial genomic DNA was extracted from fecal samples using the PowerSoil DNA isolation kit, as described by the manufacturer (MoBio Laboratories Ltd, Carlsbad, CA, USA) and samples were homogenized using a Minibeadbeater – (Biospec products, Oklahoma, USA). Bacterial 16S rRNA gene sequences were PCR-amplified from each sample by Labcycler (Sensoquest, Göttingen, Germany) using the 515F-806R universal primers; <http://www.earthmicrobiome.org/emp-standard-protocols/16s/>, containing Illumina adapter sequences to target the V4 hypervariable region of the 16S rRNA gene, including 12-base barcodes, as previously described²⁸. PCR reactions consisted of 12.5ul PrimeSTAR Max DNA Polymerase 250T- R045A (Takara, Japan), 1ul of 806R(10uM), 7.5ul of nuclease free water (hylabs, Rehovot, Israel), 2ul of 515F(5uM) and 2ul of DNA template. Reaction conditions consisted of an initial denaturing step for 3 min at 95 °C, then, 29 cycles of 10 s at 98°C, 5 s at 55°C and 5 s at 72°C, followed by 1 min at 72°C and 5 min at 4°C. Duplicate PCR reactions were performed for each sample, performed in 96-well plates and in tubes. To visualize DNA presence we used agarose Gel, which was prepared with 1.5% agarose (Benchmark scientific, New Jersey, USA) dissolved in TAE Buffer (hylabs, Rehovot, Israel) with 1 drop of Etidium Bromide (hylabs, Rehovot, Israel) for every 50 ml of TAE Buffer. For DNA loading, we used Gel loading dye (NEB, Massachusetts, USA) at the ratio of 1:6 in the favour of the sample and 100bp DNA ladder H3 RTU (GeneDirex, Miaoli, Taiwan) as a marker. Horizontal electrophoresis cells (Bio-Rad, Primefield-Landmark Building, Singapore) and MS-300V Power Supply (major science, California, USA) with voltage of 120V were used for running the DNA. The gel was captured by U.V. camera InGenius3 (Syngene , Cambridge, UK). Next, PCR products were combined and then purified with Ampure magnetic purification beads (Agencourt, Danvers, MA, USA). Purified PCR products were quantified using a Quant-iT PicoGreen dsDNA assay (Invitrogen, Carlsbad, CA, USA). Equimolar ratios (50ng) of total samples were pooled and sequenced at the Faculty of Medicine of the Bar Ilan University (Safed, Israel) using a MiSeq Sequencer (Illumina, Madison, WI,

USA). In some samples, we experienced very low DNA concentrations and on other samples, there was a loss of material in the PCR process, leading to inability to proceed with these samples. Thus, only 251 samples went further in the analysis (Fig 2).

3.2.2 16S rRNA Gene Sequence Analysis

Quantitative Insights Into Microbial Ecology (QIIME)

QIIME is an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data, developed primarily in the Knight and Caporaso labs²⁹. QIIME is designed to take users from raw sequencing data generated on the Illumina or other platforms through publication quality graphics and statistics. This includes demultiplexing and quality filtering, Operational Taxonomic Units (OTU) picking, taxonomic assignment, phylogenetic reconstruction, and diversity analyses and visualizations. QIIME has been applied to studies based on billions of sequences from tens of thousands of samples.

MacQIIME version 1.9 software package²⁹ was used for pre-processing and sequence analysis.

pre-processing

For quality filtering of raw data we used Trimmomatic 0.32³⁰, with removing reads that are shorter than 100 base pairs after trimming, along with sequences containing primer mismatches, uncorrectable barcodes and ambiguous bases. We used join_paired_ends.py script to take forward and reverse Illumina reads and join them. Filter_barcode_file.pl command was then used to discard the barcodes of the reads that were discarded in the last two steps.

QIIME combines sample de-multiplexing (dividing sequence reads into separate files for each sample), primer removal and quality filtering processes in the script: split_libraries_fastq.py, where we set the percentage of consecutive high quality base calls (*p*) to 50%, and remained the maximum number of consecutive low quality base calls (*r*), the maximum number of ambiguous bases (*n*) and the minimum Phred quality score (*q*) as default values³¹. usearch 6.1³² was utilized for chimeras (PCR artifacts) detecting using identify_chimeric_seqs.py and filter_fasta.py commands.

Sequence analysis

Sequences were assigned to OTUs using the Greengenes (GG) database by the open reference approach, by the pick_open_reference_otsu.py command, creating

an OTUs table which was PyNAST³³ aligned. After the pre-processing, the OTUs table contained 57% of the original sequences and 96% of those sequences came from close reference database.

Core diversity analysis

On our initial analysis we spotted a group of samples which had deviated greatly in their distance from the other mass of samples, a distance that we could not explain by our metadata or by other factors. We defined this group as outliers and excluded it from the analysis, remaining with 227 samples (Fig 2).

The range of counts per sample varied from 4 to 114,451. In order to standardize sequence counts across samples with uneven sampling, we randomly selected 10,000 sequences per sample (rarefaction), which kept 203 samples (Fig 2).

The rarefaction was used as a basis to compare abundances of OTUs across samples. The GG taxonomies were used to generate summaries of the taxonomic distributions of OTUs across different taxonomic levels (phylum, class, order, family, and genus).

- **Alpha diversity analysis** - We analyzed individual within samples diversity checking the acceptable alpha diversity parameters: Chao1, Observed OTUs and PD whole tree, using compare_alpha_diversity.py command.
- **Beta diversity analysis** - We used Unweighted UniFrac for between samples analysis and PCoA visualization, by core_diversity_analyses.py command.
- **Taxa plots** - We used summarize_taxa_through_plots.py command on all 49 children at baseline after rarefaction of 10,000 to receive our study taxonomy plots.
- **Distance comparison** - We used the make_distance_boxplots.py and make_distance_comparison_plots.py commands to compare phylogenetic distances within and between samples.

Paired differences - We used the script identify_paired_differences.py to explore specific microbial changes at the child's level.

The following analysis required normalization which was obtained via collapsing microbial data by mean for each child, where there were 4 measurements for each child. The command Collapse_samples.py was used for this purpose.

Levels significance

We filtered out OTUs which their total sequence count was less than 0.01% and samples which their total sequence count was less than 1,000, leaving us with 225 samples (Fig 2).

Levels_significance command we used is constructed of the commands summarize_taxa and group_significance, which provides statistical differences of the OTUs relative abundances between groups by all taxonomic levels (Level 1:Kingdom, Level 2: Phylum, Level 3: Class, Level 4: Order, Level 5: Family, Level 6: Genus, Level 7: Species). This script was written by Omry Koren's Lab, faculty of medicine, Zefad, Israel.

For the next analysis, we filtered out OTUs which their total sequence count was less than 0.01% and samples which their total sequence count was less than 1,000 (Fig 2).

Linear discriminant analysis effect size (LEfSe)³⁴

LefSe is an algorithm for High-Dimensional biomarker discovery and explanation that identifies genomic features (genes, pathways, or taxa) characterizing the differences between two or more biological conditions (or classes). It emphasizes both statistical significance and biological relevance, allowing researchers to identify differentially abundant features that are also consistent with biologically meaningful categories (subclasses). LEfSe first robustly identifies features that are statistically different among biological classes. It then performs additional tests to assess whether these differences are consistent with respect to expected biological behavior. Specifically, LefSe uses the non-parametric factorial Kruskal-Wallis (KW) sum-rank test and the unpaired Wilcoxon rank-sum test followed by Linear Discriminant Analysis (LDA) to estimate the effect size of each differentially abundant feature.

We used the Galaxy platform³⁵ for several analysis tools.

(<http://huttenhower.sph.harvard.edu/galaxy/>), with LEfSe being one of them.

In order to fit the data for LefSe, we needed to transform the data from biom format to a tabular one. The next steps are:

- LDA Effect Size: performs the analysis using the data formatted with module A and provides input for the visualization modules.
- Plot LEfSe Results: graphically reports the discovered biomarkers with their effect sizes.

- Plot Cladogram: graphically represents the discovered in a taxonomic tree specified by the hierarchical feature names.
- Plot One Feature or Differential Features: plots the row values of a feature or several features as an abundance histogram with classes and subclasses structure.

To keep the results reliable, we decided not to take into account taxonomies that were found significant by LEFSE but at the feature level analysis contained not more than 2 representative samples of the examined taxonomy in the compared groups.

Multivariate Association with Linear Models (MaAsLin)

MaAsLin is a multivariate statistical framework that finds associations between clinical metadata and microbial community abundance or function, and it is best used in the case when you are associating many metadata with microbial measurements.

We used Galaxy platform to perform maaslin analysis³⁶

Summarize_taxa.py command was used to normalize the data for MaAsLin.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)

Metagenome functional predictive analysis (including metabolic path was associated to microbes) was carried out using PICRUSt software³⁷.

PICRUSt, a computational approach to predict the functional composition of a metagenome using marker gene data and a database of reference genomes.

PICRUSt uses an extended ancestral-state reconstruction algorithm to predict which gene families are present and then combines gene families to estimate the composite metagenome.

OTU abundance was normalized by 16S rRNA gene copy number, identified and compared to a phylogenetic reference tree using the Greengenes database, and was assigned functional traits and abundance based on known genomes and prediction using the Kyoto Encyclopedia of Genes and Genomes (KEGG)³⁷.

This analysis was made on the galaxy platform as well.

- Verification of taxonomies was performed through the following database:
https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=MicrobialGenomes;

3.3 Statistical analysis

3.3.1 16S rRNA data and metadata

In order to look for associations between the metadata obtained and the abundances of the OTUs in QIIME, we employed Kruskal Wallis test (between the groups) and Monte Carlo test (within the groups) and corrected for multiple comparisons using FDR (<0.05).

In LEfSe, we used Linear discriminant analysis (LDA) score to estimate the effect size of each differentially abundant feature, with the default threshold of the LDA effect size being 2 and p-value <0.05.

In PICRUSt, Group_significance script was utilized in order to check for significance where FDR<0.05.

In MaAsLin, factor and boolean data is plotted as knotched box plots, and continuous data is plotted as a scatter plot with a line of best fit. MaAsLin performs a reduction to a linear model and uses the FDR corrected p-value (q-value) for significance.

Verifying significant results

Wherever means/abundances of OTUs differed between groups as a result of extremely high representation of a specific OTU by only a few samples, the difference was not referred as a genuine one.

3.3.2 Within metadata

For exploring the correlation within the metadata we used JMP Pro 10 software (SAS, North Carolina, USA). The statistical significance was set at p-value <0.05 at all performed tests.

For examining metadata interaction, we used baseline data only (period A) to avoid multiple identical records for each child.

3.3.3 Countinuous numeric metadata

To test countinuous numeric metadata, we applied pairwise correlations by multivariate method. Linear regression assumptions were examined for proper interpretation of the model. The assumption of normality was evaluated by Shapiro Wilk test, by symmetry of the distribution (bell-shaped, skewness, kurtosis) and by residual normal quantile plot (the normality of residuals distribution). The assumption of independence was assessed by Durbin Watson test in fit model platform.

We used matched pair t test where values in X axis and Y axis represented the same child.

3.3.4 Analysis of variance

For these analyses, several assumptions were ought to be met. The assumptions of normality and independence were examined as described earlier. The equality of variances were checked by Bartlett and Levene tests.

If all assumptions were valid, we performed oneway Analysis of variance (ANOVA) for equal variances. Alternatively, we used Welch's test when variances within groups were unequal, or non-parametric Kruskal Wallis test, when normality assumptions could not be met. For comparing means in non-parametric tests, we applied Wilcoxon non-parametric comparison for each pair.

3.3.5 Categorical metadata

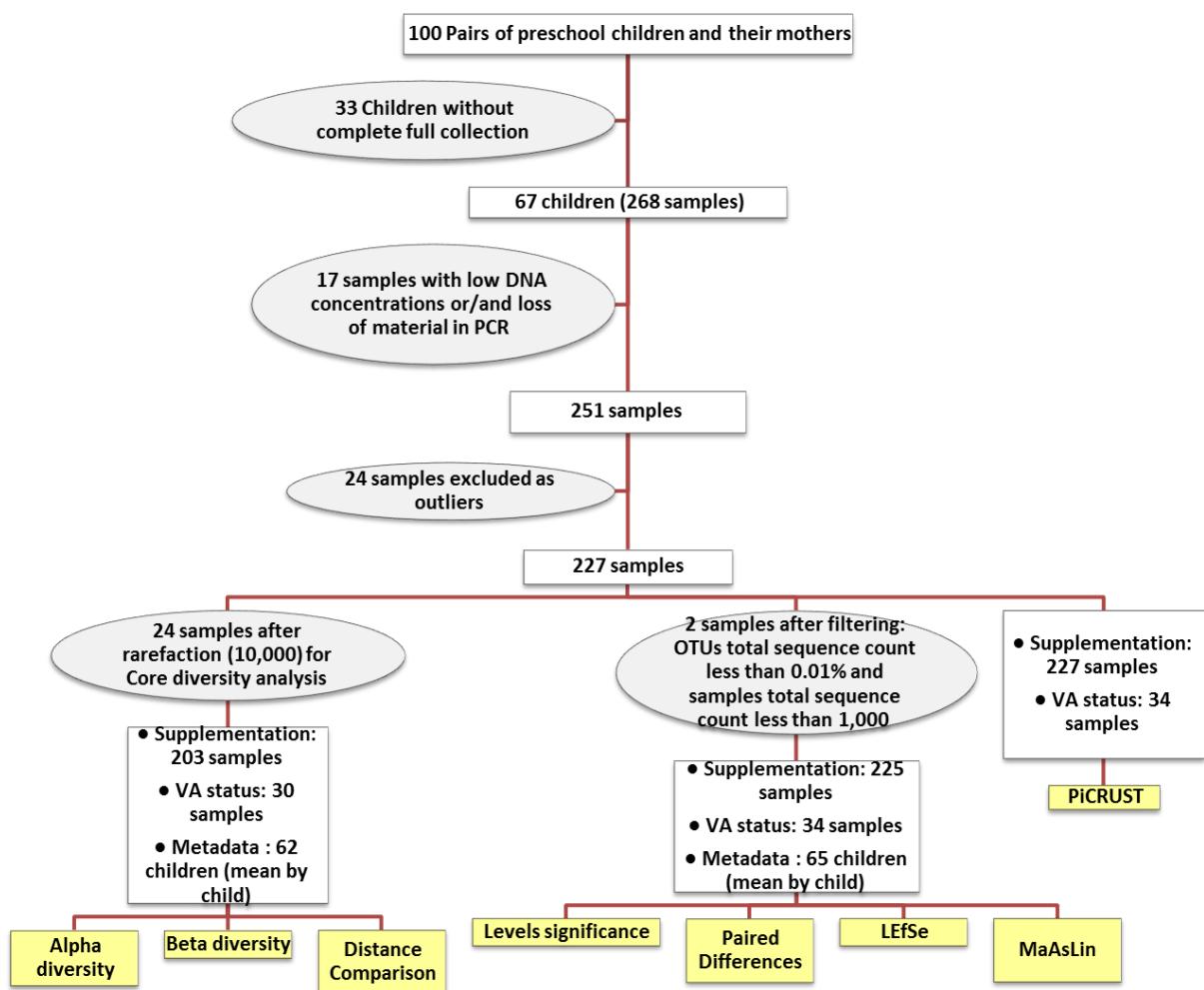
When we looked for differences between categorical metadata, we used Pearson's Chi-Square and Likelihood Ratio Chi-Square tests on the contingency modelling. If more than 20% of the cells in the contingency table had expected frequencies which are less than 5, then, to get a better approximation, we used Fischer's Exact test in JMP when the contingency table was 2 X 2 (rows X columns). More expanded tables were calculated through online algorithm;

http://www.physics.csbsju.edu/stats/exact_NROW_NCOLUMN_form.html;

3.3.6 Visualizing metadata correlations

To visualize significant findings, JMP Graph Builder, Fit Y by X platform and Excel 2010 chart platform were used.

Figure 2: Elimination process and analysis of samples



4 RESULTS

4.1 Metadata Analysis

The study population consisted of 67 children at the age of 6 months, with quite equal representation of gender. Among them, 18 were preterm children. All children were exclusively breast fed and went through one of four vaccination plan. Some children (18%) received antibiotics and almost third of all children (28.4%) went through infectious disease prior to baseline. When we observe the course of pregnancy, some mothers had gestational malaria (11.8%). All births but one were regular delivery with the mean gestational age of 38 weeks. Some mothers took antibiotics during pregnancy (10.5%) and during breastfeeding (4.5%). We made a distinction of maternal typical diet and divided the mothers into 4 groups.

Table 1: Metadata of the initial 67 children.

Child's Antropometry and Growth		
Baseline age [average in months]		6.15 ± 0.41
Gender	Male	35
	Female	32
Number of preterm children		18 (27%)
Birth weight (BW)	Birth weight (BW) [average in kg]	3.05 ± 0.6
	Number of children who had a low birth weight (LBW), BW < 2.5 kg	10 (15%)
	Birth weight percentile [average]	47.85 ± 32.56
	Number of children whose Birth Weight for age is less than 5th percentile	6 (9%)
	Number of children whose Birth Weight for age is less than 80% of the median	7 (10.5%)
Baseline weight	Baseline weight [average in kg]	7.6 ± 1.18
	Baseline weight percentile [avarage]	48.77 ± 29.62
	Number of children whose baseline Weight for age is less than 5th percentile	5 (7.5%)
	Number of children whose baseline weight for age is less than 80% of the median	3 (4.5%)
Post-natal growth	Number of children who descended at least 2 major percentiles	12
	Number of children who ascended at least 2 major percentiles	17
	Number of children without significant crossing of major percentiles	38
	Weight gain from birth to baseline [average in kg]	4.56 ± 1.02
Baseline height	Baseline height [average in cm]	67.16 ± 3.94
	Baseline height percentile [avarage]	58.25 ± 36.07
	Number of children whose baseline height for age is less than the 5th percentile height	9 (13.4%)
Baseline weight for height percentile	Baseline weight for height percentile [average]	47.28 ± 37.42
	Number of children whose baseline weight for height is less than 80% of the median	5 (7.5%)
Child's Immune System		
Exclusively Breastfed		all
Number of children who received Supplementation		none
Number of children who received Vaccinations	BCG, Polio, DPT-HepB-Hib	26
	BCG, Polio, DPT-HepB-Hib, PCV	23
	BCG, Polio, DPT-HepB-Hib, PCV, ROTA	14
	BCG, Polio, DPT-HepB-Hib, ROTA	4
Number of children who received Antibiotics	Prior to baseline	12 (18%)
	at 3 weeks of age	1
	at 4 weeks of age	7
	at 8 weeks of age	3
	at 16 weeks of age	1
Number of children who had infectious disease		19 (28.4%)
Number of children who were hospitalized		6 (9%)
Mother's Pregnancy and Delivery		
Age [average in years]		24.97 ± 6.52
Number of children per mother [average]		3.12 ± 2.64
Course of pregnancy	Number of mothers who experienced normal pregnancy	59
	Number of mothers who experienced Malaria during pregnancy	8
Delivery type	Number of mothers who experienced normal delivery	64
	Number of mothers who experienced Cesarean delivery	1
Gestational age [avarage in weeks]		38 ± 2.24
Mother's Health and Nutrition		
Number of mothers suffered of chronic disease		none
Number of mothers who took antibiotics	During pregnancy	7 (10.5%)
	at 1 week of pregnancy	1
	at 2 weeks of pregnancy	2
	at 3 weeks of pregnancy	1
	at 24 weeks of pregnancy	1
	During breastfeeding	3 (4.5%)
	at 2 weeks of breastfeeding	1
	at 3 weeks of breastfeeding	1
	at 24 weeks of breastfeeding	1
Nutrition	Number of mothers who consumed vegeterian based diet	18
	Number of mothers who consumed meat in the diet	26
	Number of mothers who consumed fish in the diet	8
	Number of mothers who consumed meat and fish in the diet	14

4.1.1 Growth Patterns of Children

According to Cole et al. the anthropometric criteria for diagnosing failure to thrive should be met on multiple occasions²⁴. Though baseline height was provided, the birth length was not, thus, we were not able to calculate the number of children whose birth height was less than 5th percentile birth height, and, consequently, we were not able to calculate birth weight for birth length percentiles. We were able to determine how many children had weighted less than 5th percentile weight for age, and, how many had weighted less than 80% of the median weight for age, both in birth and baseline (table 1). Nevertheless, in both criteria, we did not find children that fulfilled the criteria at both occasions. When we looked at the crossing of weight percentiles from birth to baseline, 12 children (18%), 9 of them were preterm infants, descended at least 2 major percentiles, implying on growth retardation. On the other hand, 17 children (25.3%), 2 of them were preterm infants, ascended at least 2 major percentiles (table 1). Children who went through retarded growth, i.e. children who descended at least 2 major percentiles, began with higher BW and BW percentile than children who went through catch-up growth, i.e. children who ascended at least 2 major percentiles (Fig 3A). Out of the children who crossed at least 2 major lines up or down, most of the preterm children experienced retarded growth, whereas, most of the regular birth children experienced catch up growth (Fig 3B). Preterm and regular children did not differ in birth weight.

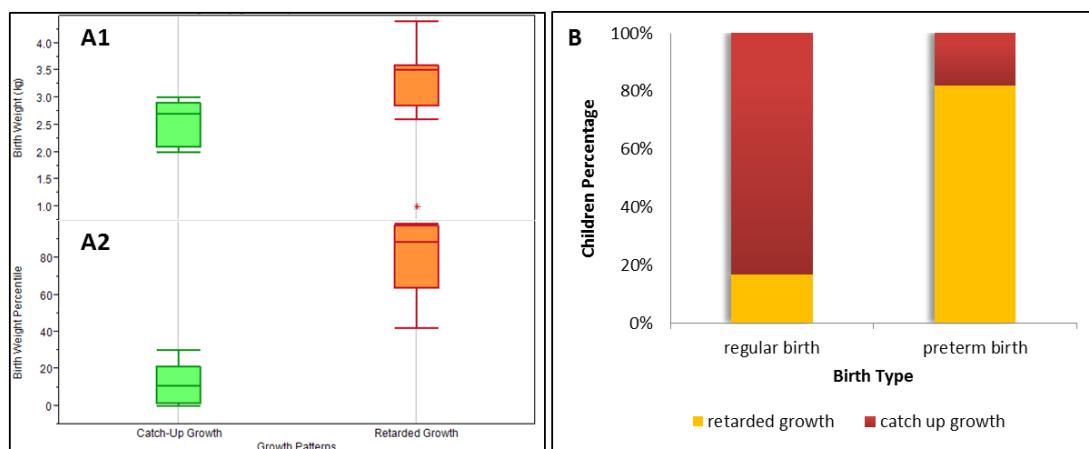


Figure 3: A1) The connection between birthweight and post-natal growth patterns (retarded vs. catch-up growth), Kruskal Wallis test, p=0.0023. A2) The connection between birthweight percentile and post-natal growth patterns, Bartlett test, p=0.017; Number of samples: Catch up growth 17, retarded growth 12. B) The connection between preterm birth and postnatal growth restriction, fisher test, p=0.0013. Number of samples: 9 out of 11 retarded growth in preterm, 3 out of 18 retarded growth in regular birth.

4.1.2 Health and Immunity of Children

Children who were hospitalized before baseline had higher baseline weight than those who were not hospitalized (Fig 4A).

Within the hospitalized children, the majority of children were with malaria (fig 4B).

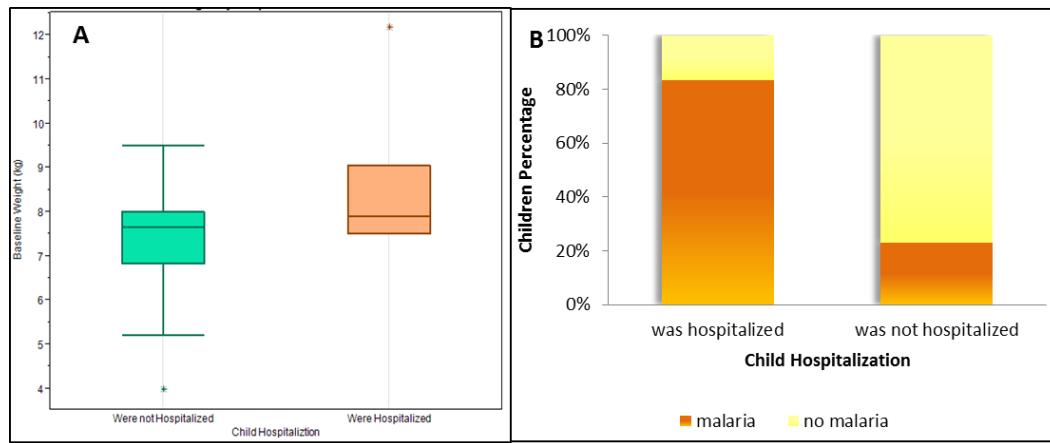


Figure 4: A) The effect of Hospitalization of children on their Baseline weight, ANOVA, p=0.048; Number of samples: hospitalized 6, not hospitalized 60. B) The connection between hospitalization of children and Malaria, fisher test, p= 0.006. Number of samples: 5 out of 6 had malaria within the hospitalized, 14 out of 61 had malaria within the not hospitalized.

Children who received antibiotics before baseline had experienced larger weight gain (between birth and baseline) and had reached higher baseline weight and weight percentile than those who did not receive antibiotics fig 5A).

Proportion of children who had malaria was higher among children who received antibiotics than among those who did not fig 5B).

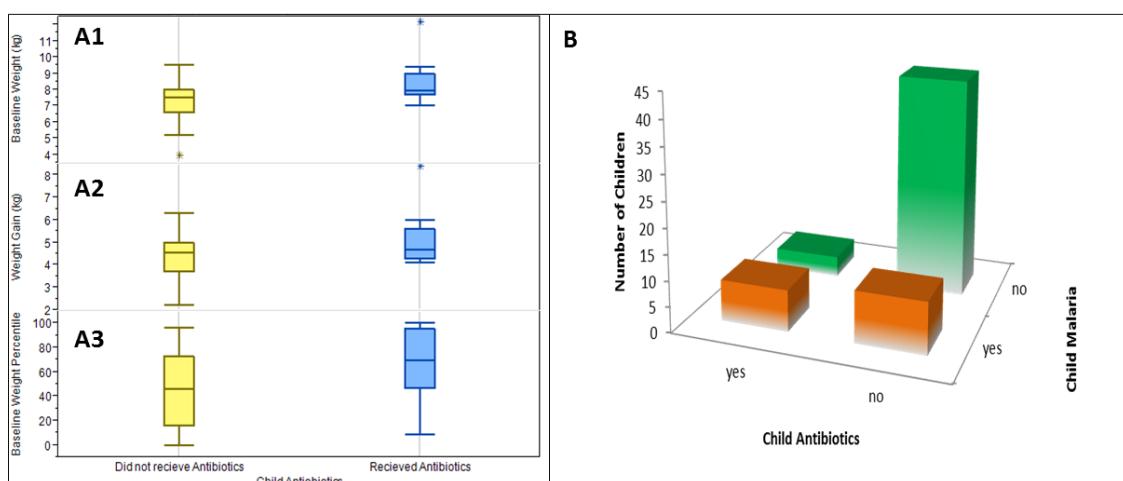
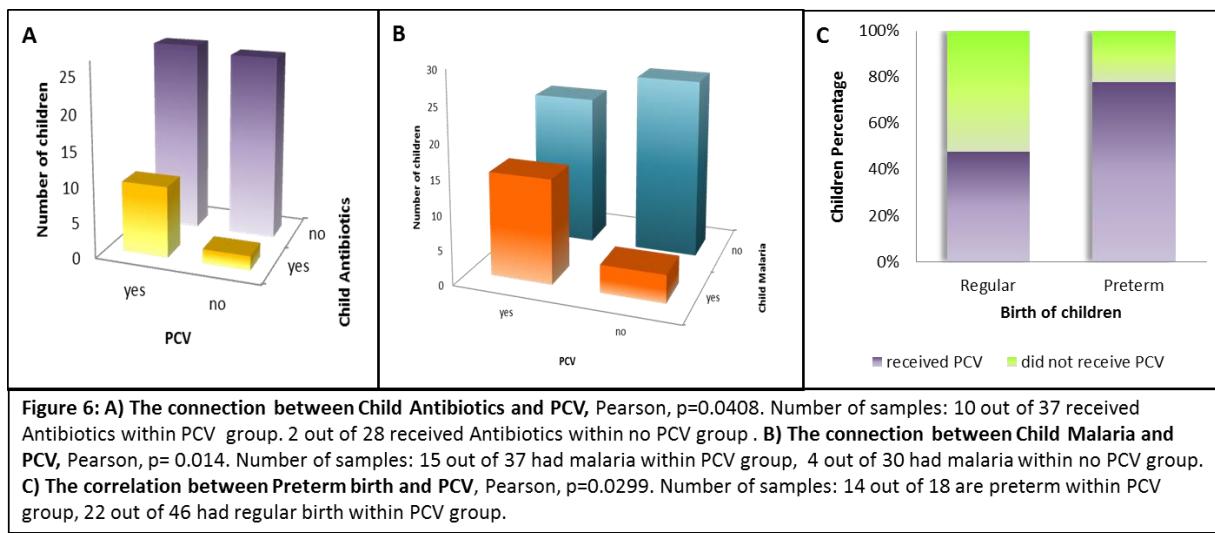


Figure 5: A1) The effect of child antibiotics on their Baseline weight, ANOVA, p=0.0059; A2) The effect of child antibiotics on their Weight gain, ANOVA, p=0.00349; A3) The effect of child antibiotics on their Baseline weight percentile, ANOVA, p=0.0112; Number of samples: Antibiotics 12, no Antibiotics 52. B) The connection between Child Antibiotics and Malaria, Fisher test, p= 0.0021; Number of samples: 8 out of 10 had malaria within Antibiotics group, 10 out of 53 had malaria within no antibiotics group.

The proportion of children who received antibiotics was higher among PCV recipients than among those who didn't receive PCV. Fig 5A)

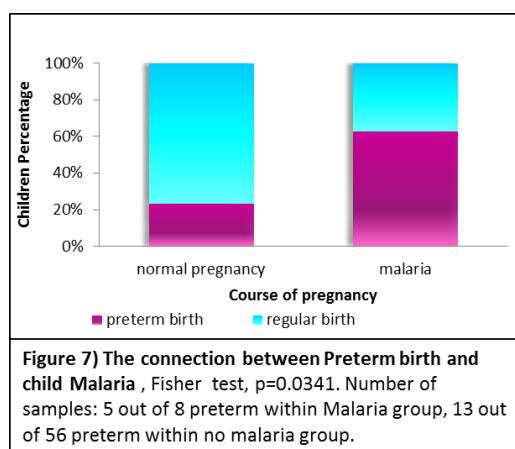
The proportion of children who had malaria was higher among PCV recipients than among those who didn't receive PCV(fig 6B).

There was higher percentage of PCV recipients among preterm children than among non-preterm ones (fig 6C).



4.1.3 Maternal Health and Nutrition

The group of mothers who suffered from Malaria during pregnancy, experienced higher percentage of preterm birth (fig 7).



We found there were trendily more preterm children to the group of mothers who consumed meat compared to those who did not, with significance only when comparing less than 37 weeks to 39-40 weeks (data not shown).

Within the group of children who did not undergo malaria, there was higher percentage of mothers whose diet included fish (fig 8A).

Children of mothers who ate meat, compared to those who did not, had higher percentage of antibiotics usage (fig 8B).

There was a higher percentage of hospitalized children among children whose mothers ate vegetarian diet, compared to children whose mothers did not eat vegetarian diet (Fig 8C).

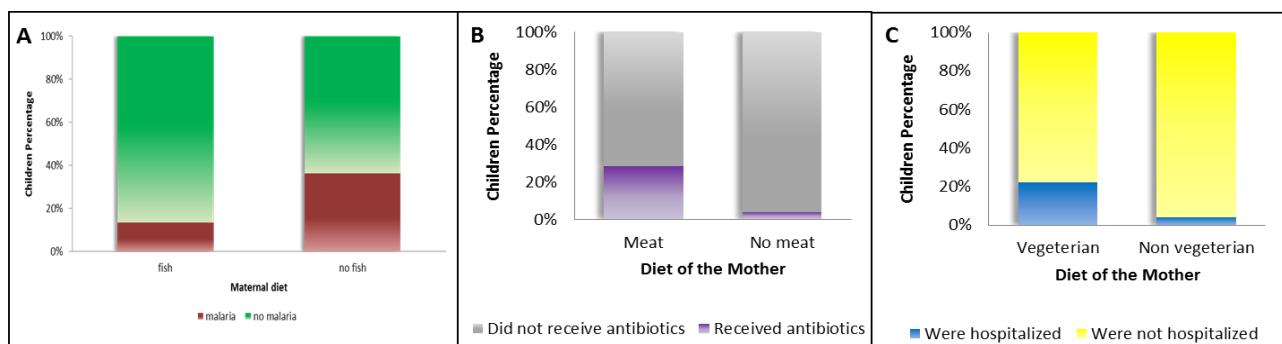


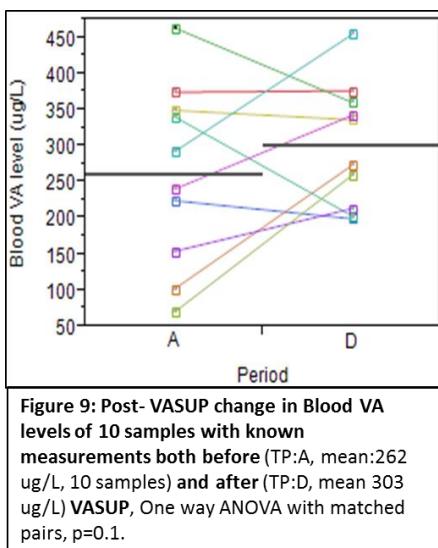
Figure 8: The interaction between maternal diet and child health outcomes A) The connection between maternal Fish consumption and child Malaria, Pearson , $p=0.05$. Number of samples: 3 out of 22 had malaria within the fish group, 16 out of 44 had malaria within the no fish group. B) The connection between maternal Meat consumption and Child antibiotics, Fisher test, $p=0.02$. Number of samples: 11 out of 39 received antibiotics within the meat group, 1 out of 25 received antibiotics within the no meat group. C) The connection between maternal vegetarian diet and child Hospitalization, Fisher, $p=0.043$. Number of samples: 4 out of 18 hospitalized within Vegetarian, 2 out of 48 hospitalized within non vegetarian.

4.2 Effect of VASUP on VA blood levels

Out of 40 children checked for VA blood levels in TP:A and 26 children checked for those levels in TP:D, only 10 children had been tested in both TPs, after we excluded one child from the analysis because one of his samples was severely hemolytic (table 2), and it could interfere with the analysis²⁵. When we matched VA blood levels of those children, we witnessed an elevated trend after VASUP (fig 9). At a group level, the average of VA levels of all children in TP:D was also trended higher than all in TP:A (table 2). Moreover, there was an increase in the percentage of VAS children following VASUP (70% to 90%), and consequently, a decrease in the percentage of VAD children (30% to 10%) (table 2). As there were only 3 children who were VAD in baseline, out of the 10 children with VA measures for both TP:A and TP:D, we were not able to statistically assess how baseline VA status of the children affects the VA blood level after VASUP.

Table 2: Summary of VA blood levels of the 67 children

	Children with known VA levels in either A (before VASUP) or D (a month after VASUP) or both				Children with known VA levels in both A (before VASUP) and D (a month after VASUP)			
Period	A		D		A		D	
Number of children with known VA level	40		25		10			
VA status	VAS	VAD	VAS	VAD	VAS	VAD	VAS	VAD
Number of children (%)	31 (73%)	9 (23%)	20 (80%)	5 (20%)	7 (70%)	3 (30%)	9 (90%)	1 (10%)
VA level average and SD [ug/L]	334 ± 16	92 ± 14	320 ± 20	174 ± 18	327 ± 31	110 ± 24	313 ± 27	200
	279 ± 19		291 ± 24		262 ± 34		303 ± 34	



4.3 Correlation between VA blood levels and other metadata

Here we present correlations between blood VA levels of children and several metadata characteristics. As the children have a better VA status, their weight and weight percentile at 6 months of age is higher (fig 10A).

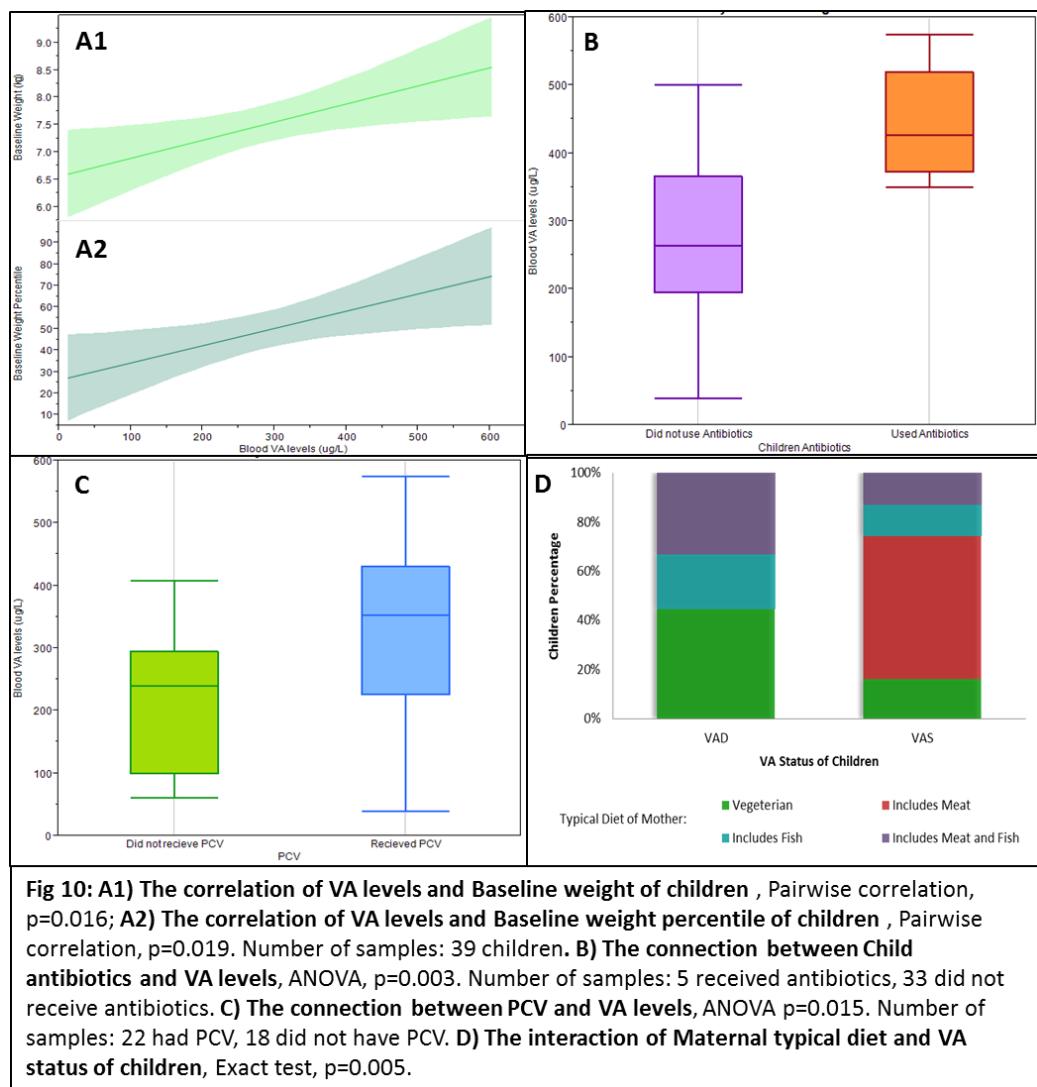
We discovered that children who used antibiotics reached a higher baseline weight (fig 10B).

When we observed at gestational age of mothers, we noticed a different, yet not significant, distribution of VAD and VAS children by gestational age. For the sake of statistics, we omitted the only VAD child who was born at 26 weeks. All children born in late preterm (34-36 weeks) were VAS. There was higher proportion of VAS than

VAD children in the early term group (37-38 weeks), but lesser than in the term group (39-40 weeks).

Children who received PCV vaccine had higher VA level at 6 months old than those who did not (fig 10C).

We found a different distribution of maternal diet between VAD and VAS groups. There were no VAD children to mothers who ate meat but not fish. There was a higher percentage of children within vegetarian maternal diet among VAD compared to VAS children (fig 10D).



4.4 Correlations between 16S rRNA data and metadata

4.4.1 Taxonomy of study population

The microbiome population of our study consisted of 4 major phylas (fig 11, table 3): *Actinobacteria* (48.6%), *Firmicutes* (27.1%), *Proteobacteria* (16.3%) and *Bacteroidetes* (7.4%). *Bifidobacterium* Genus constituted almost all of *Actinobacteria* Phylum (47%). Within the *Firmicutes* phylum, *Clostridiales* was the main order (18%), and afterwards *Lactobacillales* (9.1%). The prominent genera of the *Clostridiales* were *Veillonela* (13.7%), *Clostridium* (0.7%) and *Blautia* (0.6%). *Streptococcus* (6.2%) and *Lactobacillus* (2.3%) were dominant among the *Lactobacillales*. The phylum *Proteobacteria* was chiefly composed of the family *Enterobacteriaceae* (15.7%). The *Bacteroidetes* mostly included the families *Prevotellaece* (4%) and *Bacteroidaceae* (2.5%).

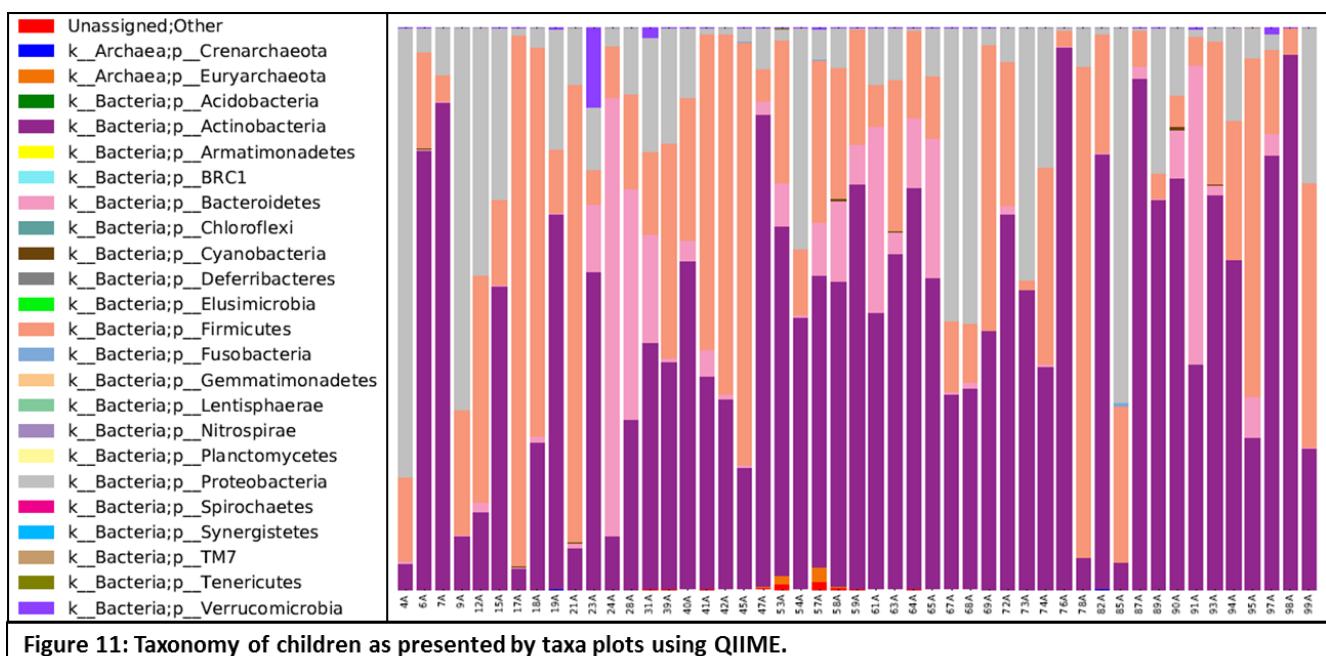
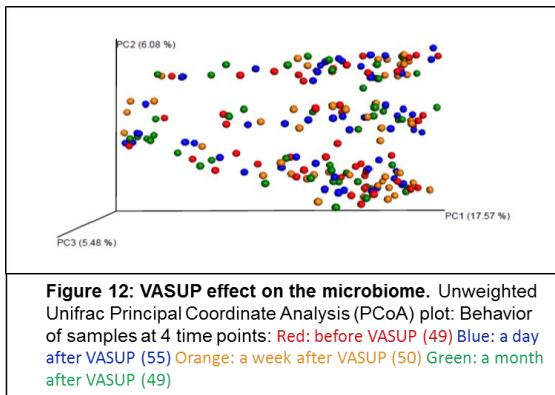


Table 3: Taxonomy of Study Population

Phylum	Class	Order	Family	Genus
Actinobacteria 48.6%				
	Actinobacteria 47.2%			
		Bifidobacteriales 47%		
			Bifidobacteriaceae 47%	
				Bifidobacterium 47%
		Actinomycetales 0.4%		
			Actinomycetaceae 0.1%	
				Actinomyces 0.1%
			Micrococcaceae 0.1%	
				Rothia 0.1%
	Coriobacteria 1.4%			
		Coriobacterales 1.4%		
			Coriobacteriaceae 1.4%	
				Collinsella 0.2%
Firmicutes 27.1%				
	Clostridia 18%			
		Clostridiales 18%		
			Veillonellaceae 14%	
				Veillonella 13.7%
				Megasphaera 0.1%
			Clostridiaceae 1.5%	
				Clostridium 0.7%
			Lachnospiraceae 1.5%	
				Blautia 0.6%
				Coprococcus 0.2%
				Clostridium 0.1%
				Dorea 0.1%
				Ruminococcus 0.1%
			Tissierellaceae 0.4%	
				Peptoniphilus 0.2%
				Gallicola 0.1%
			Ruminococcaceae 0.2%	
				Faecalibacterium 0.1%
			Peptostreptococcaceae 0.1%	
			Eubacteriaceae 0.1%	
				Pseudoramibacter
				Eubacterium 0.1%
	Bacilli 9.1%			
		Lactobacterales 9.1%		
			Streptococcaceae 6.2%	
				Streptococcus 6.2%
			Lactobacillaceae 2.3%	
				Lactobacillus 2.3%
			Enterococcaceae 0.6%	
Proteobacteria 16.3%				
	Gammaproteobacteria 15.9%			
		Enterobacterales 15.7%		
			Enterobacteriaceae 15.7%	
				Citrobacter 0.3%
		Pasteurellales 0.2%		
			Pasteurellaceae 0.2%	
				Haemophilus 0.2%
	Epsilonproteobacteria 0.3%			
		Campylobacterales 0.3%		
			Campylobacteraceae 0.3%	
				Campylobacter 0.3%
	Alphaproteobacteria 0.1%			
Bacteroidetes 7.4%				
	Bacteroidia 7.4%			
		Bacteroidales 7.4%		
			Prevotellaceae 4%	
				Prevotella 0.4%
			Bacteroidaceae 2.5%	
				Bacteroides 2.5%
			Porphyromonadaceae 0.4%	
				Parabacteroides 0.4%
			Paraprevotellaceae 0.2%	
				YRC22 0.1%
				Prevotella 0.1%
			Barnesiellaceae 0.1%	
Verrucomicrobia 0.4%				
	Verrucomicrobiae 0.4%			
		Verrucomicrobiales 0.4%		
			Verrucomicrobiaceae 0.4%	
				Akkermansia 0.4%

4.4.2 VASUP and the Microbiome

We were interested to see if there were different cluster of the samples at the 4 TPs, yet the samples does not appear to behave differently (fig 12).

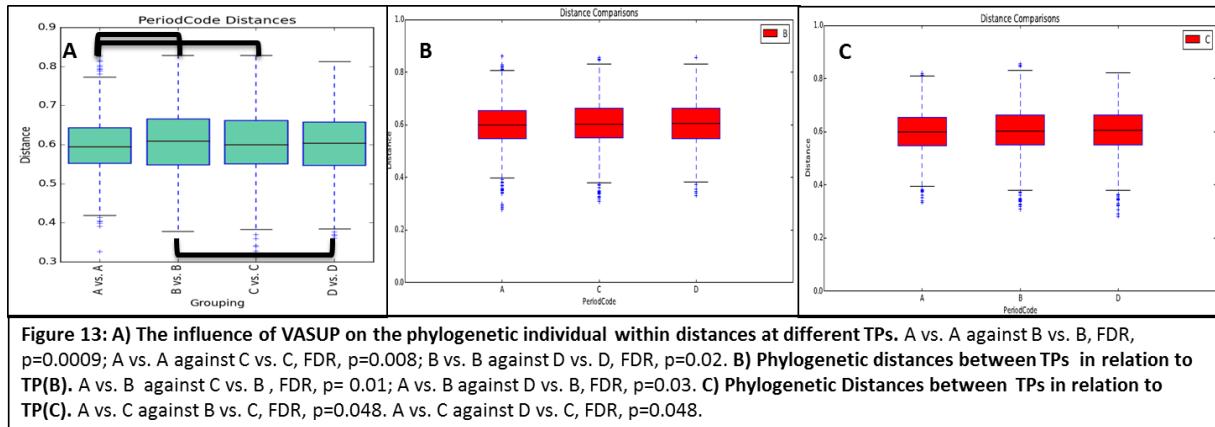


We explored the phylogenetic distances of the microbiome in our 4 TPs at a group level, i.e. each TP represents a group of children.

First, when we examined the individual within distances, we found there has been a significant difference in the distances of the microbial community between TP:A and TP:B (FDR, $p=0.0009$), and also, between TP:A and TP:C (FDR, $p=0.008$). In addition, we discovered significance when comparing distances of TP:B and TP:D (FDR, $p=0.02$) (fig 13A).

Second, we compared distances between all TPs, in relation to one TP at a time. When comparing the TPs in relation to TP:B, we revealed that the distance of TP:A from TP:B is significantly different from the distance of TP:C from TP:B (FDR, 0.01), and, the distance of TP:A from TP:B is significantly different from the distance of TP:D from TP:B (FDR, 0.03) (fig 13B). Moreover, when the TPs in relation to TP:C, we revealed that the distance of TP:A from TP:C is significantly different from the distance of TP:B from TP:C (FDR, 0.05), and, the distance between of TP:A from TP:C is significantly different from the distance of TP:D from TP:C (FDR, 0.05) (fig 13C). Yet, when comparing TPs in relation to TP:A or to TP:D, we saw no significance.

Interestingly, when we examined whether there were differences in specific OTUs at a group level, following VASUP, we did not get significant results, not even when we tested sub-group such as VA status at baseline or receiving PCV vs. not receiving (see below).



Not less important, we were interested to see if there were paired differences of OTUs followed by VASUP at a child level. Likewise, we have not found significance, not even by the sub-groups mentioned above.

Lastly, when we performed metagenome functional predictive analysis, we have not seen functional change followed by VASUP.

4.4.3 VA Status and the Microbiome

Next, we were interested to see if VA status of the children influences the microbiome. Specific OTUs significantly differed by VA status of the children at baseline (fig 14, 15).

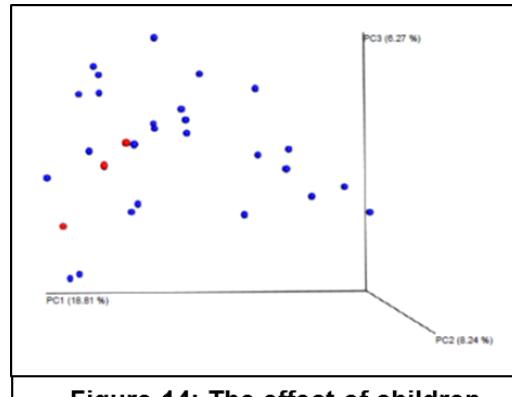
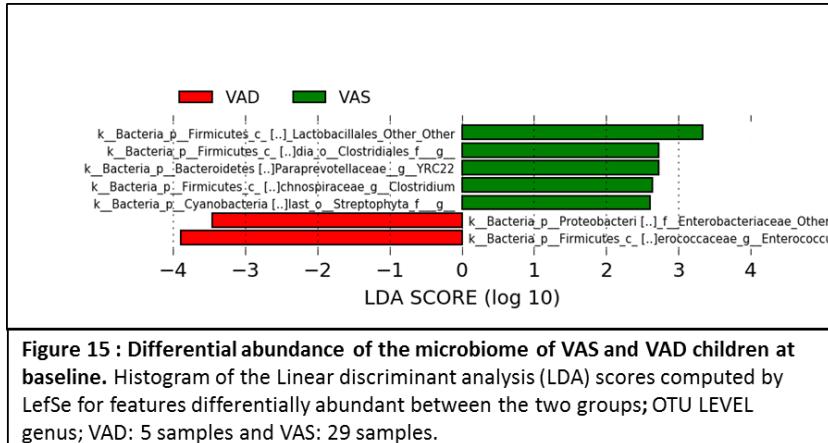
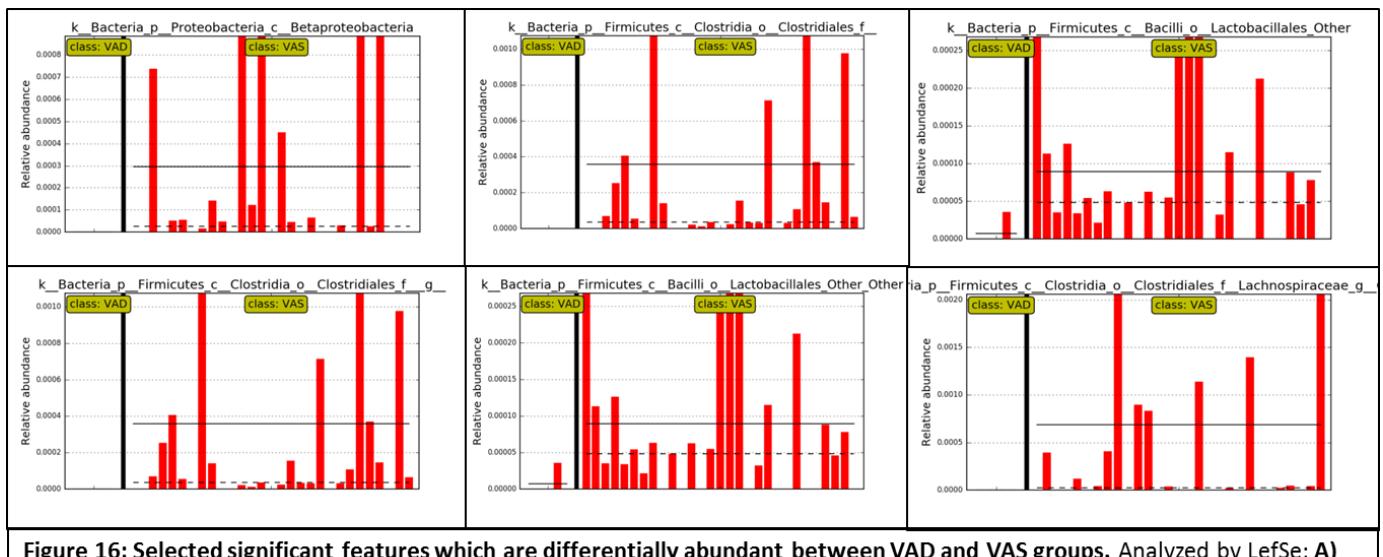


Figure 14: The effect of children VA status on their microbiome.
Unweighted Unifrac Principal Coordinate Analysis (PCoA) plot;
Blue: VAS (26) Red: VAD (3)



The class of *Betaproteobacteria* was more abundant among VAS children in comparison to VAD children. The *Clostridiales* order was more represented in the VAS group than the VAD one. Within this order, mainly the *Lachnospiraceae* family and the *Clostridium* genus were more abundant in the VAS group. Members of the Order *Lactobacillales* were more abundant in the VAS group (fig 16). We discovered no differences when comparing the individual within and between distances of VAD vs.VAS children at baseline, and neither when predicting metagenome functions.



4.4.4 Other Metadata and the Microbiome

As we approached to check the interaction of other metadata with the microbiome, we normalized the differences between TPs by collapsing the data under each child, i.e. referring to the mean of all TPs.

4.4.4.1 PCV and the Microbiome

The major metadata characteristic to differ in the microbiome composition was PCV. We found alpha diversity differences, in all 3 parameters, between children who received PCV to those who did not (fig 17).

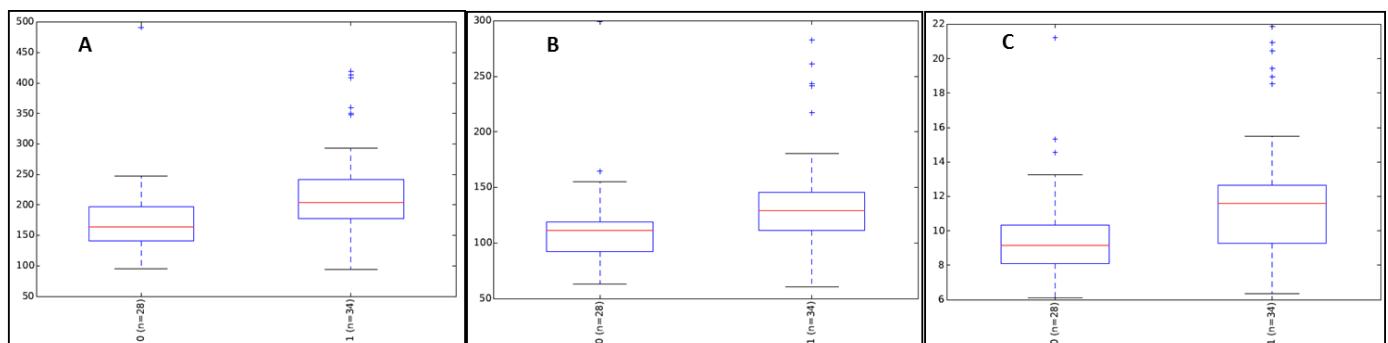
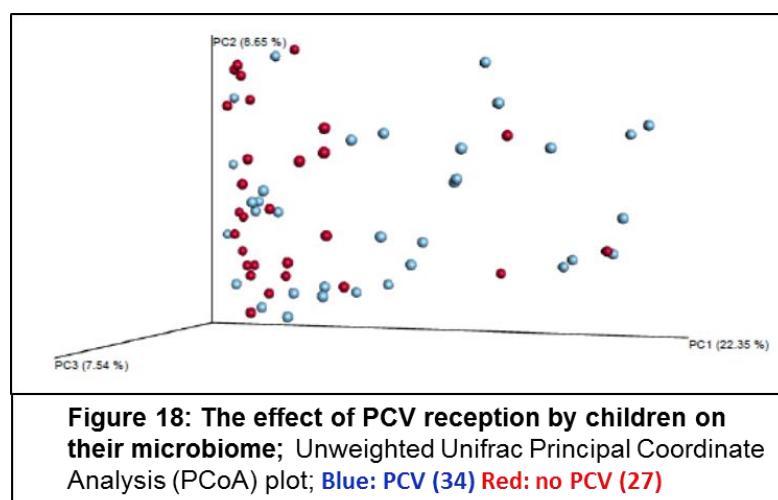


Figure 17: Comparison of Alpha Diversity, Phylogenetic within Diversity of the microbiome (branch length-based diversity), between children who received PCV and children who did not; A) Chao1, p=0.027 B) Observed OTUs, p=0.02 C) PD whole tree, p=0.013; 0=no PCV, 1=PCV.

When exploring beta diversity differences, we also found different microbiome patterns between PCV groups (fig 18).



We further investigated the specific OTUs differences of those groups and discovered numerous distinctions at the OTU level (fig 19).

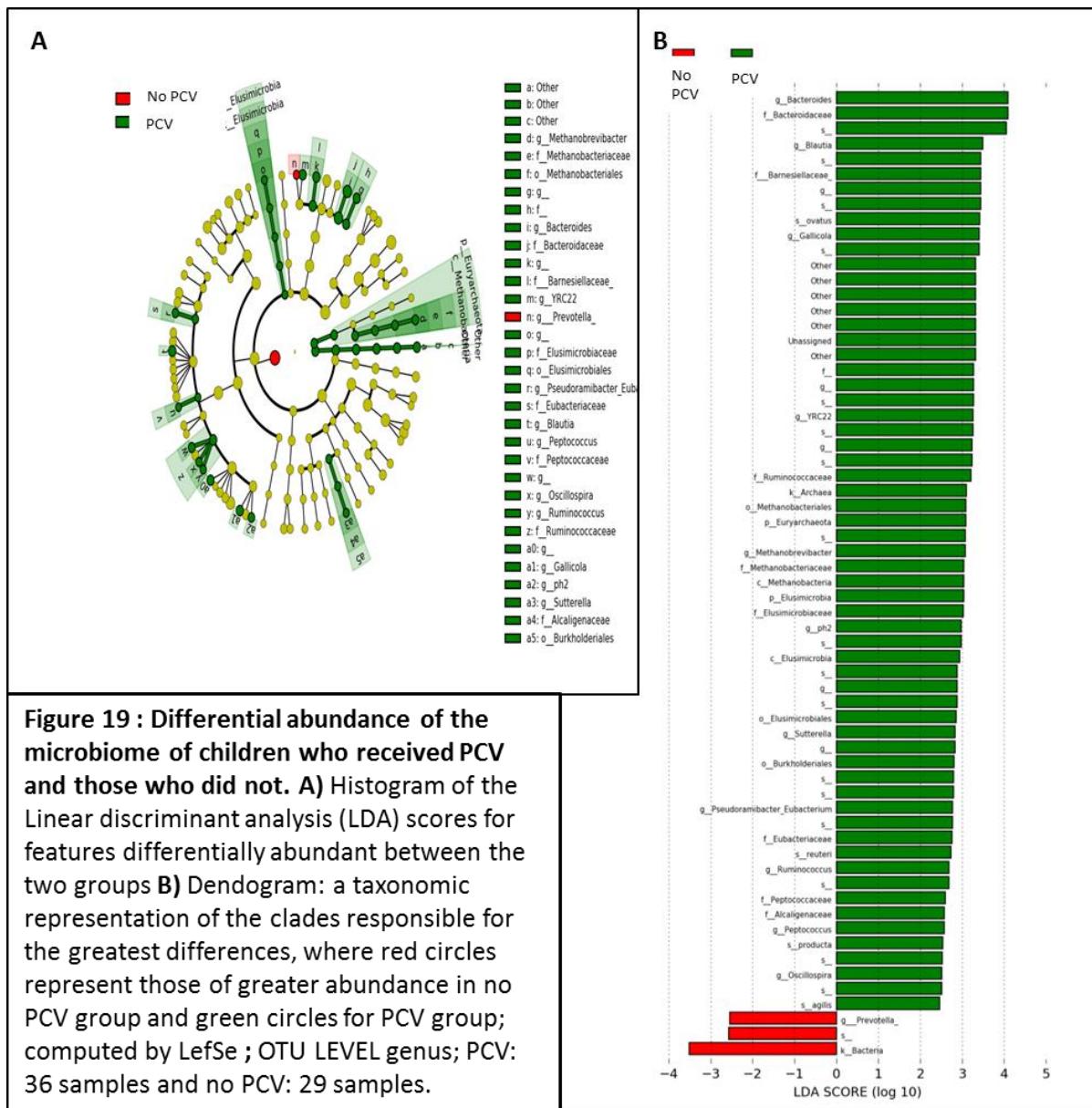


Figure 19 : Differential abundance of the microbiome of children who received PCV and those who did not. A) Histogram of the Linear discriminant analysis (LDA) scores for features differentially abundant between the two groups B) Dendrogram: a taxonomic representation of the clades responsible for the greatest differences, where red circles represent those of greater abundance in no PCV group and green circles for PCV group; computed by LefSe ; OTU LEVEL genus; PCV: 36 samples and no PCV: 29 samples.

There were OTUs represented more at the PCV group (fig 20). At the family level: *Peptococcaceae* family and unspecified family of *Bacteroidales*; At the genus level: *Blautia* genus, *Ruminococcus* genus, *Peptococcus* genus, unspecified genus of *Veillonellaceae*, unspecified genus within *Clostridiales*, unspecified genus of *Ruminococcaceae*, unspecified genus of *Barnesiellaceae*; At the species level: *Lactobacillus agilis* species, *Blautia producta* species, unspecified species of *Veillonellaceae*, Unspecified species of *YRC22*, Unspecified species of *Blautia*, Unspecified species of *Ruminococcus*, Unspecified species of *Barnesiellaceae*, Unspecified species of *Bacteroides*, Unspecified species of *Bacteroidales*,

Unspecified species of *Bacteroidia*, Unspecified species of *Peptococcus*, Unspecified species of *Pseudoramibacter Eubacterium*, Unspecified species of *Gallicola*.

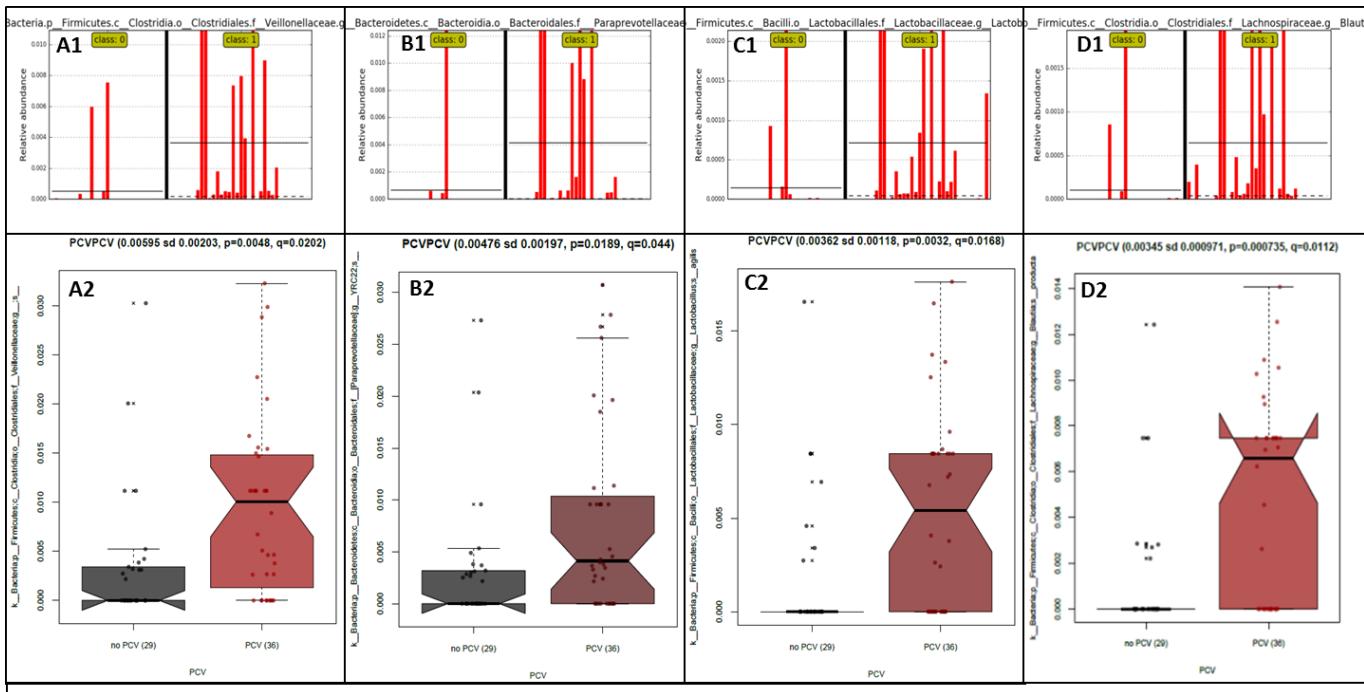


Figure 20 (part 1): Selected significant features which are differentially abundant between children who received PCV and those who did not (LefSe) and were tested for Multivariate Association with Linear Models (MaAsLin); A1-2) Unspecified species of *Veillonellaceae* family B1-2) Unspecified species of *YRC22* genus C1-2) *Lactobacillus agilis* D1-2) *Blautia producta*; class: 0 = no PCV, class: 1 = PCV.

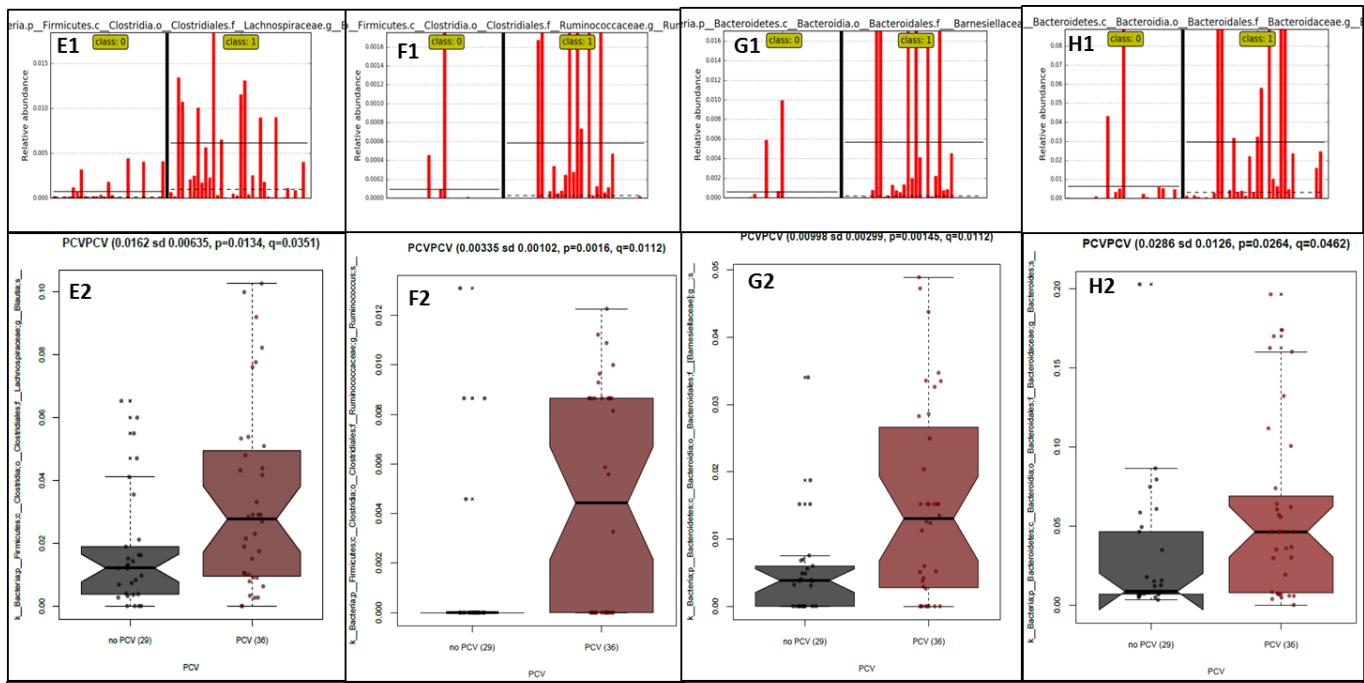
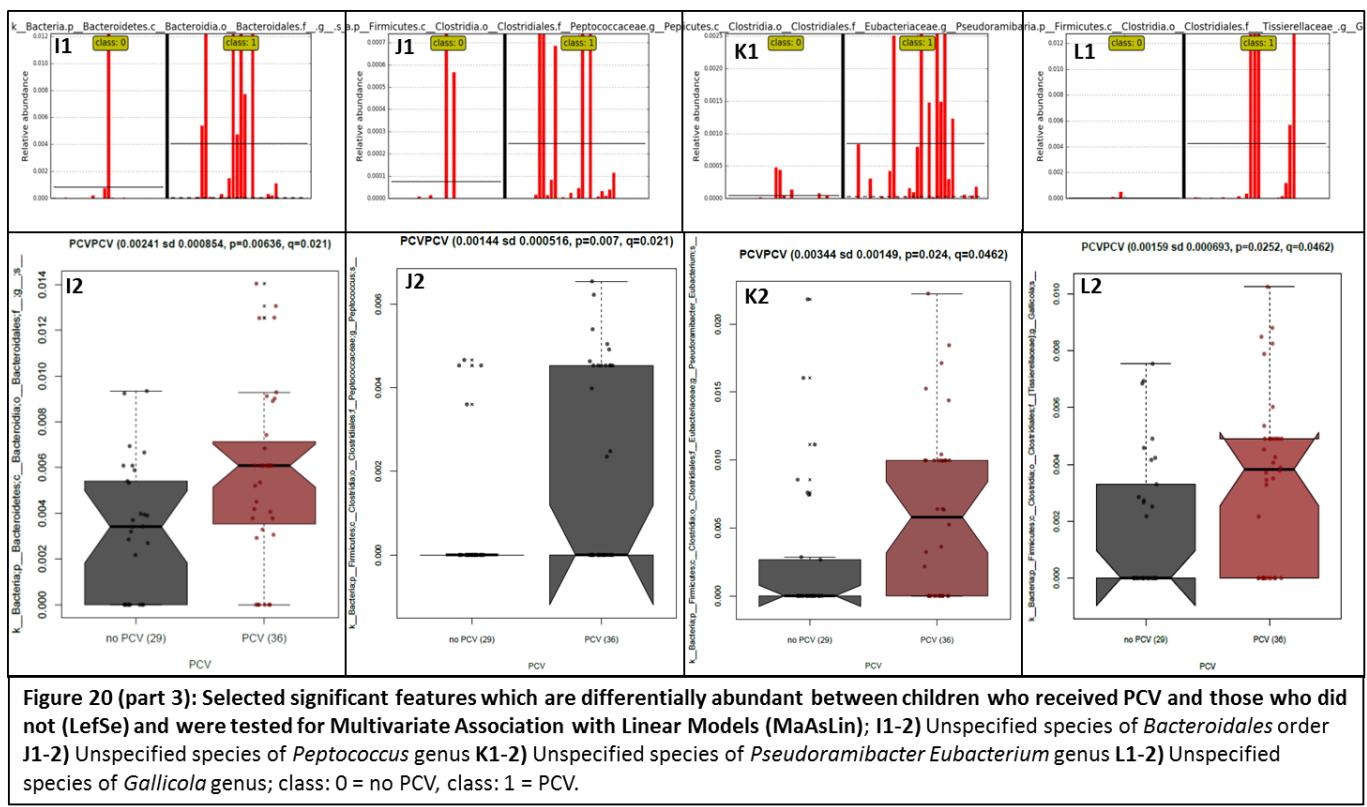
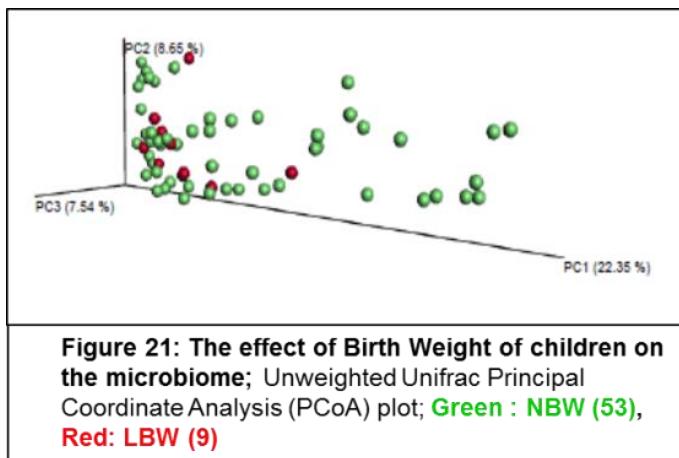


Figure 20 (part 2): Selected significant features which are differentially abundant between children who received PCV and those who did not (LefSe) and were tested for Multivariate Association with Linear Models (MaAsLin); E1-2) Unspecified species of *Blautia* genus F1-2) Unspecified species of *Ruminococcus* genus G1-2) Unspecified species of *Barnesiellaceae* family H1-2) Unspecified species of *Bacteroides* genus ; class: 0 = no PCV, class: 1 = PCV.

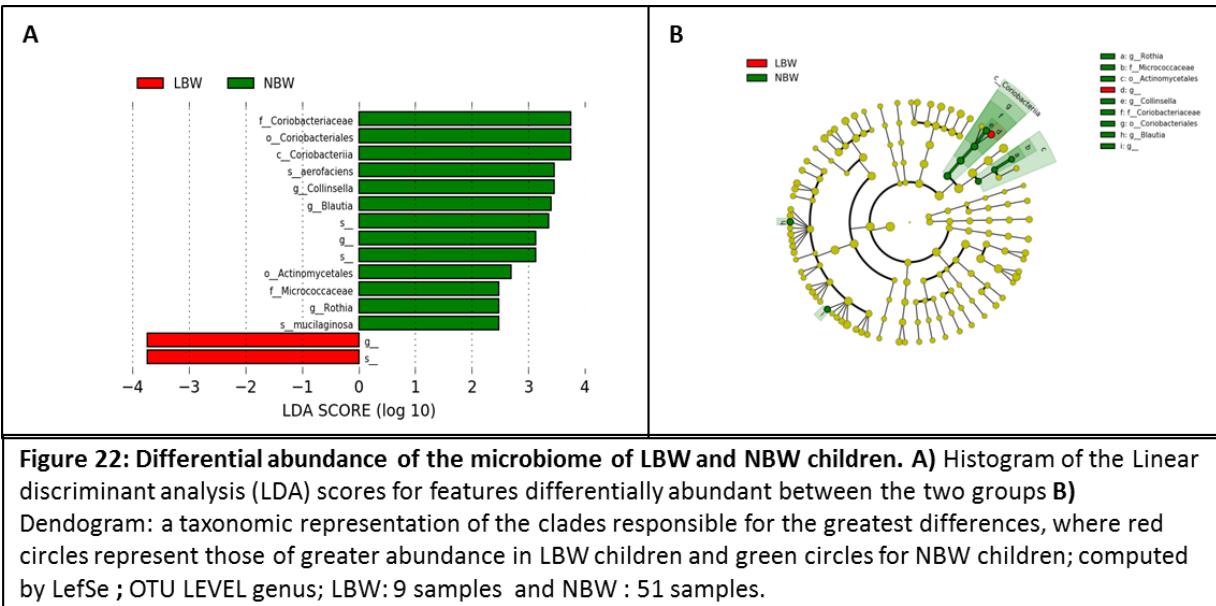


4.4.4.2 BW and the Microbiome

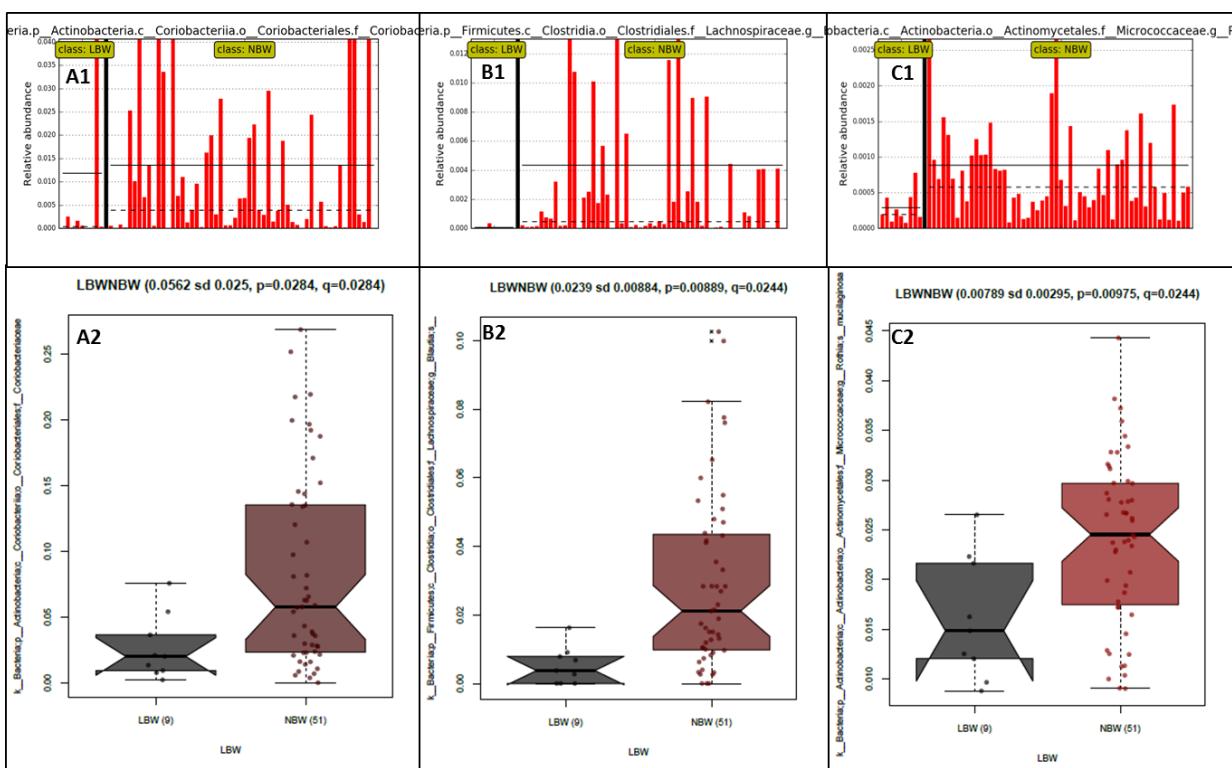
When we examined birth weight of the children, we found beta diversity difference between the groups (fig 21).



We analyzed distinctions at the OTU level and found significant differences between the NBW and LBW groups (fig 22).

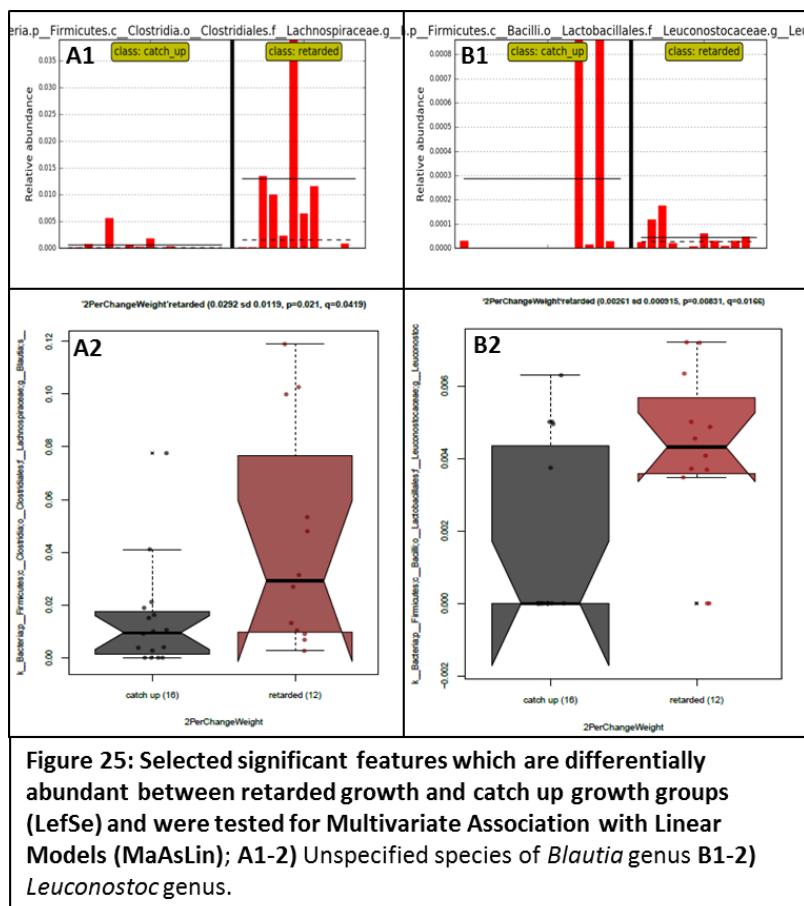
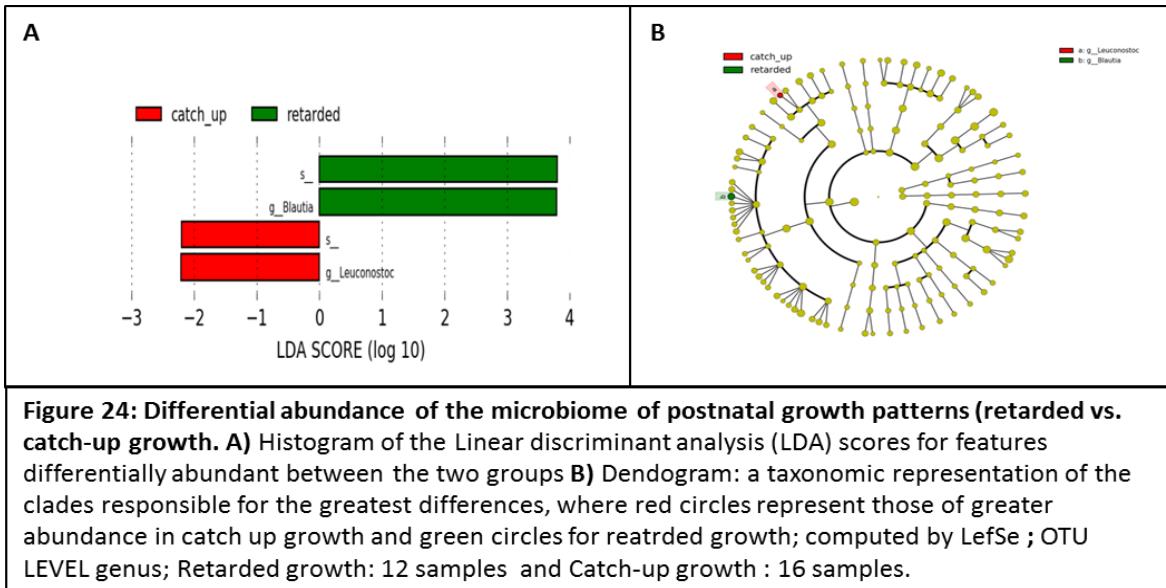


There were OTUs represented more at the NBW group than in the LBW group (fig 23). At class level: *Coriobacteriia*; At the order level: *Coriobacteriales*, *Actinomycetales*; At the family level: *Coriobacteriaceae*, *Micrococcaceae*; At the genus level: *Blautia*, *Rothia*; At the species level: Unspecified species of *Blautia*, *Rothia mucilaginosa*.



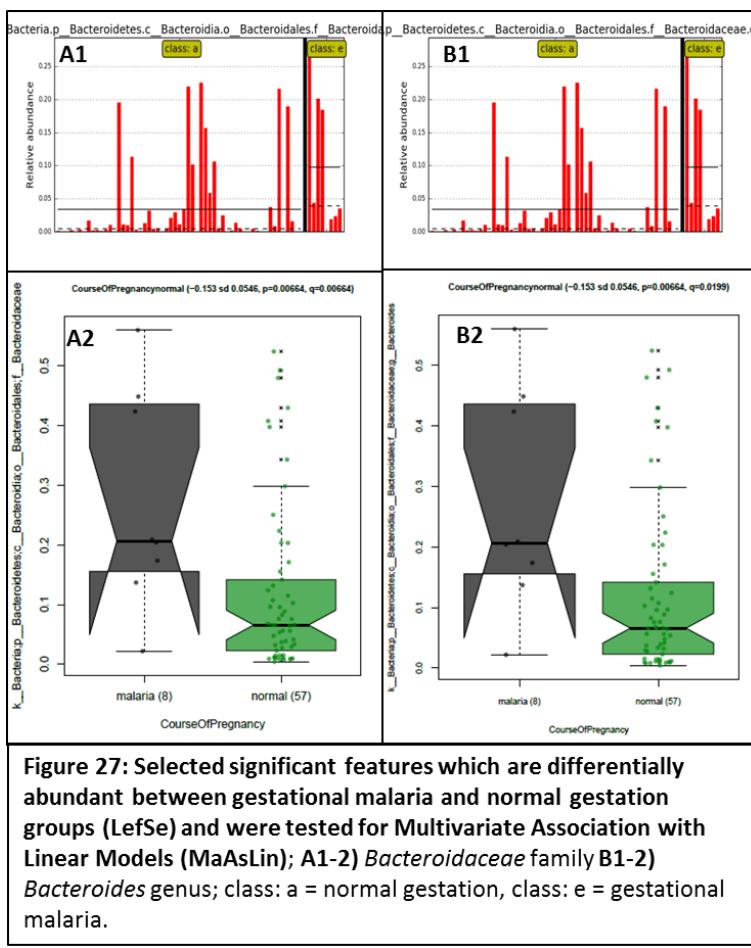
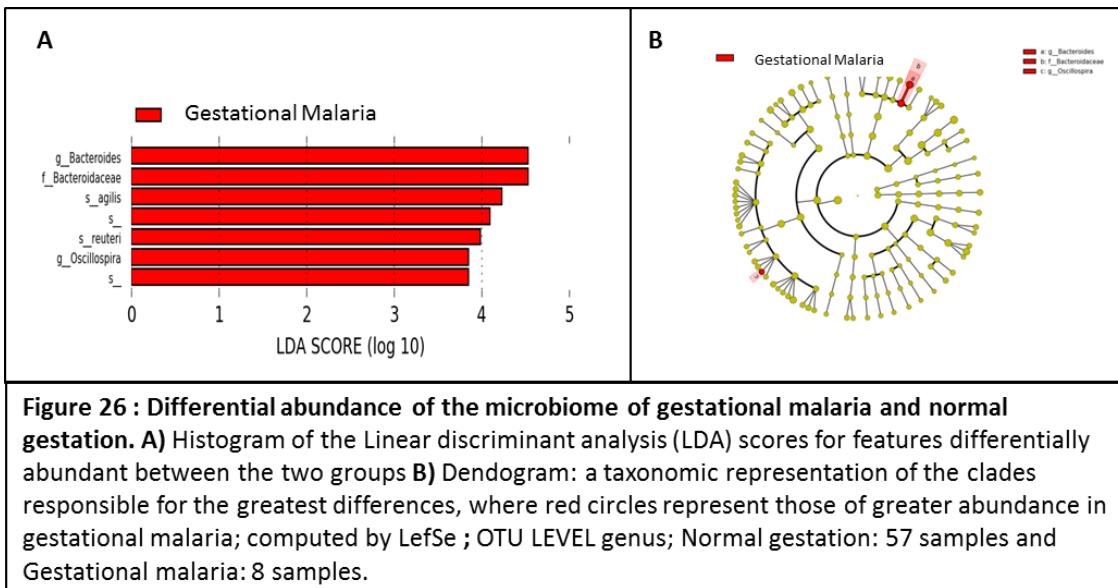
4.4.4.3 Growth Patterns and the Microbiome

We found a connection between the pattern of post-natal growth and the relative abundance of some OTUs (fig 24). *Leuconostoc* genus and unspecified species of *Blautia* are more represented in the retarded growth group (fig 25).



4.4.4.4 Gestational Malaria and the Microbiome

We revealed that *Bacteroidaceae* family and *Bacteroides* genus were represented more in mothers who had malaria during pregnancy than those who had normal pregnancy (fig 26,27).



5 DISCUSSION

The aim of this work was to reveal microbiome difference following VASUP. Secondly, to explore microbiome changes as dependent of VA status. Thirdly, to discover potential metadata features which affect microbiome. Lastly, to find connections of interest within metadata characteristics.

5.1 Metadata Analysis

5.1.1 Growth, Health and Immunity of children

We found a connection between preterm birth and postnatal growth restriction. Postnatal growth restriction, similar to our findings, is very common among preterm infants and might be associated with neurodevelopment impairment.³⁸ On the other hand, upward curve of postnatal growth in preterm infants has also been described, in contrast to growth flattening in cross-sectional birth charts³⁹.

We showed that hospitalized infants have higher baseline weight. Hospital stay has an impact on the nutritional status of children. Opposed to our study, studies show that one of the risk factors for hospital acquired malnutrition is hospital stay duration 5 days or more⁴⁰. Hospitalized children exposed to high nutritional risks have poor clinical results and greater weight loss^{41,42}. Children with a low BMI Z-score on admission showed a BMI decrease at the end of their hospital stay. Despite that, there is now substantial global experience of strategies and interventions to improve the quality of care for children in hospitals with limited resources through tools that have been field-tested by doctors, nurses and other child health workers in many developing countries. As a result, countries with limited resources and other major obstacles, achieved improvements in quality of pediatric care. One of these countries is Malawi where these changes were accompanied by a 40% reduction in inpatient mortality over 2 years^{43,44}.

We revealed that the majority of the hospitalized infants were due to malaria. When we look for the major cause of child hospitalization we find that in Uganda the prevalence of children malaria is very high, around 50% of the admitted children⁴⁵⁻⁴⁷.

We discovered a connection between antibiotics and larger weight gain. Like our study, other works showed that antibiotic exposure before 6 months of age was consistently associated with increased body mass, increase in weight-for-length later in infancy^{48,49} but not through age 7 years⁵⁰.

We found an association between malaria and antibiotic usage. A connection between malaria and antibiotic usage has been described in literature. Some antibiotics are considered to be anti-malarial drugs⁵¹. Moreover, in a nationwide sample of malaria patients presenting to health facilities in Uganda, over-prescription of antibiotics was extremely common.⁵² From another point of view, a recent study identified the presence of antibiotics in the blood of malaria-infected people as a new risk of increasing disease transmission. The investigators showed that antibiotics in ingested blood enhance the susceptibility of *Anopheles gambiae* mosquitoes to malaria infection by disturbing their gut microbiota. Antibiotic exposure additionally increases mosquito survival and fecundity, which are known to augment vectorial capacity. Thus, malaria transmission may be exacerbated in areas of high antibiotic usage⁵³.

We showed a correlation between PCV recipients and antibiotic usage. Our finding regard PCV and antibiotics stands against the claim that PCV reduces incidence of disease caused by antibiotic-resistant bacteria, and through this, reduced antibiotic use⁵⁴.

We revealed a connection between preterm infants and higher reception of PCV. Preterm infants are at increased risk for pneumococcal disease, particularly infants born earlier than 32 weeks of gestation, and this explains why we found a high preterm proportion among PCV recipients. The increased risk may be due to reduced transfer of pneumococcal antibodies to fetus and a decreased response to *Streptococcus pneumoniae* because of an immature immune system^{55,56}.

5.1.2 Maternal Health and Nutrition

In our finding, gestational malaria was associated preterm birth. Malaria in pregnancy is one of the major causes of mortality and morbidity in tropical regions, causing preterm birth, among other outcomes.⁵⁷ Several studies show the association of maternal persistent malaria with increased risk of preterm birth⁵⁸, especially late preterm (34–36 weeks)⁵⁹. To support our result, specific Ugandan prospective cohort, which dealt with the impact of malaria during pregnancy on pregnancy outcomes, found that the risk of pre-term delivery was increased in mothers with malaria in pregnancy occurring within two weeks of delivery⁶⁰.

We discovered a connection between preterm birth and maternal meat consumption. Several foods, nutrients, dietary supplements, and more recently dietary patterns, have been investigated in relation to risk of preterm delivery, based on their potential influences on antioxidant defense, inflammation, immunity, insulin sensitivity, blood

pressure, and blood lipids.⁶¹ One study showed increased odds of preterm birth by a diet characterized by a high consumption of red meats, fried chicken and processed meats⁶². Other study suggested that western-type diet, high in meat and fats and low in fruits and vegetables, is associated with increased odds of induced preterm birth⁶³. Our discovery joins to these findings.

We found an association between malaria of children and maternal fish consumption. There is an evidence of dietary fish oils as a protection against malaria and this could explain why there are less malarial morbidity among mothers who ate fish. One aspect deals with fish oil-based diets fed to mice. Studies found that these diets afford significant protection against malaria parasites⁶⁴⁻⁶⁶. Under these dietary conditions, especially omega 3 fatty acids, mice were able to control and even survive malaria⁶⁷⁻⁶⁹. Another aspect of this manner is the ability of some edible fish to reduce mosquito quantity within water sources in Africa^{70,71}.

We revealed a correlation between maternal vegetarian diet and higher hospitalization rates of children. Maternal diet during pregnancy contributes to infant health. Recent study showed that individuals in the least healthy quartile had a lower adherence to the plant-based diet received by FFQ⁷². Dietary intakes of women from the mother-offspring cohort study in Singapore were ascertained at 26–28 weeks of gestation using 24-hour recalls and 3-day food diaries. The healthier mothers were more likely to meet recommendations for intakes of total fruits, whole fruits, total vegetables, dark green leafy and orange vegetables⁷³. This contradicts our claim of more hospitalization of children among vegetarian mothers.

5.2 Effect of VASUP on VA blood levels

We found that the average of VA blood levels of children was trendily higher following VASUP.

The evidence of the effect of VASUP on VA blood levels is heterogeneous. Early controlled studies of VAD children in Thailand and Indonesia found no significant effect of high-dose VASUP on VA blood levels after 10-12 weeks^{74,75}. A later Indonesian study also reported no significant immediate impact on the mean VA levels of children who received VASUP⁷⁶. A Zambian trial, involving 155 children with measles, found no significant difference in mean VA levels two weeks after a single dose of VASUP⁷⁷. More surveys from Zambia showed little difference in VA measures at approximately 5 and 18 weeks following high-potency VASUP⁷⁸.

On the other hand, not a few studies showed significant and mixed results regarding the increase in VA levels. Data from Brazil, indicated a substantial rightward shift in blood VA distribution among children with low apparent liver stores at baseline, 5 weeks post-VASUP. However, the effect among children with adequate baseline liver stores was negligible⁷⁹. Another smaller measles trial with in Africa reported a significantly higher VA level of children on day 8, but not on day 42, after VASUP⁸⁰. A large scope trial of approximately 2580 pre-school children in north India found that mean plasma VA was one-sixth higher following VASUP⁸¹. A new study involving 64 children, aged 1 to 8 years old, with autism spectrum disorder, found their plasma VA levels significantly increased after 6 months of VASUP⁸².

If we observe how VASUP changed the proportions of VAS and VAD children, there is a resemblance between our finding and the current reports. The Brazilian study mentioned earlier described a decrease in the proportion of VAD children at 1 month following VASUP⁸³. The previous Indonesian study also showed that the proportion of VAD children 1 month following VASUP was reduced by approximately 15%⁷⁶. Furthermore, to substantially support that, the Indian trial reported that the prevalence of severe VAD was halved due to VASUP⁸¹.

Serum Retinol as indicator of VA status

It has been suggested that serum retinol is not a reflective indicator of VA status or VASUP program performance. Due to the fact that VA is homeostatically controlled in the plasma, when we come to asses VA level of children, we can, on the one hand, potentially miss out subclinical deficiency, and on the other hand, wrongly overlook the contribution of VASUP to improve the VA tissue status, while not necessarily increasing serum retinol values⁴. On the top of that, it is hard to establishment whether low serum retinol is due to insufficient intake or due to the temporary effects of infection, protein-energy malnutrition or accompanying inflammation, as RBP, VA's carrier, is a negative phase responder⁶. Overall, experimental data suggest that high-potency VASUP protects children aged 6–59 months from hyporetinolaemia for a short period of time, usually 2 months, before it returns to pre-supplementation levels⁴. While the duration of protection may be even shorter among children who are VAD at baseline, a larger dosage of VASUP may sustain adequate retinol concentrations for a longer period of time^{84,85}. Therefore, when interpreting serum retinol distributions with respect to population VA status, it needs to be taken into account that these concentrations may be negatively influenced by confounders, especially in regions with high infection burden. Given the slight ability of high-potency VASUP to raise and sustain a serum retinol distribution for longer period of

time⁴, retinol levels may not properly reflect of VASUP effect on VA status, and the question how best to measure this impact, still remains.

5.3 Correlation between VA and other Metadata

5.3.1 VA and Growth of Children

We discovered that proper VA status is associated with better growth characteristics. VAD may be associated with poor growth. Children in whom VAD is truly growth-limiting would be expected to respond to VASUP⁸⁶. Children with VAD are often stunted and occasionally wasted compared with apparently normal children of similar age⁸⁶⁻⁸⁸. Current evidence shows that VASUP of children of a low socioeconomic status increased mean weight for age and height for age, implying that VASUP may prevent growth problems⁸⁹. When considering growth velocity, children who were in a state of chronic VAD, gained less weight and height than children who were sub-clinically deficient⁹⁰.

Infants with extremely LBW(≤ 1 kg) have low plasma and tissue concentrations of vitamin A^{86,91,92}, because they have low stores of the vitamin at birth, minimal intake during feedings for several weeks or longer after birth, and poor enteral absorption of the vitamin⁹³. Several clinical trials of VASUP for VLBW infants revealed that many infants had low serum retinol concentrations and that VASUP increased their survival rate⁹⁴⁻⁹⁶. Additionally, a protective association was observed between maternal dietary intake of vitamin A and small gestational age.⁹⁷

We revealed a different, yet not significant, distribution of VAD and VAS children within different gestational ages. It has been suggested that gestational age must be taken into account when interpreting VA levels because of significant correlation the two⁹⁸. Numerous studies suggest that a poor VA status is one of the features associated with a higher prevalence of prematurity and intrauterine growth retardation found in poorly nourished populations⁹⁹. Furthermore, significant correlations were found between cord serum vitamin A, maternal serum vitamin A, gestational age, and growth status^{100,101}. Generally, preterm infants are born with inadequate body stores of vitamin A and are prone to diseases of the eye and respiratory and gastrointestinal tracts. They have low plasma concentrations of both retinol and RBP at birth compared with term infants. Plasma concentrations of retinol remain low throughout the first year of life^{102,103}. In addition, evidence shows that renal losses of retinol decrease over the neonatal period, and appear to be inversely related to gestation¹⁰⁴. However, several studies found that serum VA concentrations tend to decline slightly with gestational age. Liver concentrations of vitamin A, on the

other hand, seem to increase until about the 28th week and then to decrease appreciably to term, with the claim that the transfer of vitamin A across the placenta cannot keep pace with the fetal growth rate during the last trimester¹⁰⁵.

5.3.2 VA and Immunity of Children

We discovered a connection between antibiotics usage and higher baseline VA level of children. Early studies revealed that the amount of VA in the liver and serum carotenoids were lower in groups without antibiotics, such as penicillin and aureomycin, than those with it, indicating a sparing action of antibiotics on vitamin A and its precursors^{106,107}. Later study shows the capacity of some antibiotics, especially tetracyclines, to stimulate additional growth in rats receiving a sub-optimum intake of VA, and supports the mode of action of certain antibiotics in the economy of VA¹⁰⁸.

We found a connection between PCV reception and higher VA levels. A correlation between VA and vaccines has been described in several studies. Generally, some show that VAD impaired antibody responses and vaccine efficacy, and that VASUP improves cellular immune responses¹⁰⁹⁻¹¹¹. Others claim that VA status and/or VASUP effect differed between children who went through vaccines than those who did not¹¹²⁻¹¹⁴ and some works deal with that combination, also regarding pneumococcal polysaccharide antigen¹¹⁵.

Pneumococcal pneumonia disease claims millions of lives annually and occurs when gram-positive *Streptococcus pneumoniae*, a commensal of the nasopharyngeal tract, turns pathogenic and invades the lungs. VA status alterations were observed in response to pneumococcal pneumonia since VAD subjects have the inability to make protective antibodies and they lose lung epithelial integrity¹¹⁶.

5.3.3 VA and Maternal Diet

We found a connection between maternal diet and VA status. There was association between maternal meat consumption and VAS children and between maternal vegetarian diet and VAD children. The connection between VA consumption and VA status of both mother and child has been described in literature. The main sources of VA are fish liver oils, such as cod, and mammalian liver, such as of calves, ox, lambs or chicken. Moderate sources are milk, butter, cheese and eggs^{117,118}. Non-farmed fish, naturally occurring living resources, make a larger contribution to VA intakes¹¹⁹. Moreover, dark green leafy vegetables, yellow fruits, orange roots and the oils of palms are the main sources of pro-vitamin A. In a few studies, VAD was found to be significantly higher in children on a vegetarian diet^{120,121}. Since it has been suggested that maternal and infant concentrations of VA are highly correlated¹²², maternal VA status could probably reflect children VA status. One research, dealt

with women diet, revealed that women who consumed 3 servings or less of dark green leafy vegetables and fruit per week had significantly lower VA levels than those who consumed 4 or more servings a week. In addition, women who never consumed sweet pumpkin had significantly lower serum VA than the women who ate at least one serving a week¹²³. Similar to our finding, a Brazilian research claimed, that no consumption of animal meat by mothers is a risk factor for VAD of their infants¹²⁴.

5.4 Correlations between Microbiome and other Metadata

5.4.1 General Taxonomy of Children Population

We were intrigued to see if our findings (table 3) match the current research of infant microbiome. In our study, *Bifidobacterium* genus dominates the microbiome of infants, as in numerous other similar universal studies^{125,126}.

It has been shown that *Streptococcus* and *Lactobacillus* take a distinguished part of the infant microbiota¹²⁵, and indeed we show a relatively high prevalence of both: 6.2%, 2.3%, respectively. Members of *Corynobacteriales*, mainly *Collinsella* species, and *Actinomyces* species are among the first established species in the gastrointestinal tract of the infant¹²⁷, and our results support that (table 3).

Verrucomicrobia, which we detected in our study, is another phylum of the core microbiome. We found that *Enterobacteriaceae*, which has been described as one of the early colonizers¹¹, is highly prevalent among the infants(15.7%). Also, members of the *Lachnospiraceae* family have a respected part in the gut microbiome of infants¹²⁸, as reflected in our study.

Latest studies present microbiome differences based on geography and ethnicity of infants. A study regarding Malawian, Amerindian and US infants show higher representation of *Bacteroides*, *Prevotella* and *Streptococcus* genera among non-USA infants. Malawians and Amerindians were also distinguished from each other, e.g. *Enterococcaceae* family were overrepresented in Amerindian infants¹²⁵. Other research involving Malawian and Finnish 6 months old infants show a greater proportion of *Bifidobacteria* in Malawian than in Finnish infants. Additional distinctions in microbiome composition comprised *Prevotella* genus and certain species of *Clostridium*¹²⁶. The microbiome composition, identified at 15 weeks of age, in a study on Bangladeshi infants was quite similar to ours with a main difference in *Proteobacteria* genera and the presence of *Staphylococcus*¹¹. A research on African-American, Hispanic and Caucasians infants study also showed that race is associated with microbiome composition whereas White race was associated with lower diversity but higher *Bacteroidetes* abundance⁹. A late review focuses on a trend in the human microbiome evolution as the human populations passed through

three stages of subsistence like foraging, rural farming and industrialized urban western life. In general, gut microbiome of the hunter-gatherer populations is highly abundant with *Prevotella*, *Proteobacteria*, *Spirochaetes*, *Clostridiales*, *Ruminobacter* etc., while those of the urban communities are often enriched in *Bacteroides*, *Bifidobacterium*, and *Firmicutes*¹²⁹. Moreover, Confirming the importance of *Prevotella* as a discriminatory taxon, recent studies showed that this genus was present in higher abundance in the microbiota of children living in Africa compared with children living in Europe^{125,129}. The relative abundance of taxa of infants in rural Ghana differed in *Proteobacteria* and *Firmicutes* proportions from our findings¹³⁰.

5.4.2 VASUP and the Microbiome

In our case, we witnessed subtle influence of VASUP on the composition of the microbiome within individuals and not at the group level. Previous studies also described this effect and explained it by large variability of the infant group at baseline that is analogous to human genetic variability, in which variation among populations is small compared to variation within populations¹²⁵. Only lately, some evidence about the effect of VASUP on the microbiome has started to emerge. It has been demonstrated that VA has an inhibition effect on virus replication both in vitro and in vivo, directly or indirectly via microbiome changes, particularly of *Lactobacillus* strains in the gut.¹³¹ Another study showed that intake of beta-carotene equivalents by adults correlated negatively with *Bacteroides* and its corresponding higher level taxa and positively correlated with *Firmicutes* and specific taxa belonging to this phylum.¹³² The recent study which mentioned before, regarding children with autism spectrum disorder, found differences in gut microbiota before and after 6 months of VASUP in the subset of 20 children. It described a decrease in *Bacteroidetes/Bacteroidales* and an increase in *Bifidobacterium* proportions.⁸²

Even though we tried to keep the homogeneousness of the participants and the setting as much as we could, we found that the microbiome was effected by other features in our metadata, so it was challenging to come into conclusions on the sole effect of VASUP on the microbiome. As presented earlier, metadata characteristics such as VA baseline status, PCV, pre and post-natal growth and gestational malaria had an impact on the microbiome.

It has been proved that microbiome naturally changes overtime, especially in the first years of life¹²⁵. Although the shift we witnessed in the microbiome during a month can be attributed to the factor of time, the change within a week, and surely within a day, should be referred to the supplementation effect.

Breast milk, immune system and microbiome

Studies show that breastfeeding is a major factor that influences infant microbiome, so we believe that could mask the impact of VASUP on the microbiome^{133,134}. Mother milk's non-digestible oligosaccharides support the growth of both *Bacteroides* and *Bifidobacterium spp.* in breast-fed infants. A recent study showed that *Ruminococcus gnavus* is an exclusive representative of the *Lachnospiraceae* family in 2-months old breast-fed infants.¹²⁷ A study on infants from Italy show that breast-fed infants have a microbiota dominated by *Bifidobacterium* with a lower presence of *Escherichia coli*, *Clostridium difficile*, *Bacteroides fragilis* group, and *lactobacilli* than the microbiota of exclusively formula-fed infants.¹²⁸ In a study on White Caucasian and South Asian infants, several genera within *Firmicutes* were associated with breastfeeding. Some were more abundant, such as *Veillonella* and *Megasphaera*, with the former being abundant in our study as well (13.7%). Others were less abundant, e.g. *Blautia*, *Lachnospiraceae*, *Clostridium* and *Ruminococcus*.¹⁰ Other study involving African-American, Hispanic and Caucasians infants found that Breast-fed infants had lower proportions of *Clostridiales*⁹.

5.4.3 VA Status and the Microbiome

We discovered that VA status of infants at baseline influences the composition of the microbiome with some bacteria of interest that were more abundant at the VAS group: *Betaproteobacteria*, *Clostridiales*, *Lachnospiraceae* and *Lactobacillales*.

Betaproteobacteria

Some members of this class cause diseases in humans. It has been shown that although VA positively affects antibodies production, it does not influence antibodies production when it comes to disease caused by some *Betaproteobacteria* members.¹³⁵ Other study shows that VA promotes phagocytosis of a genus of this class.¹³⁶ Overall, we could not find a rational in that finding.

Clostridiales* and *Lachnospiraceae

Research shows that *Clostridiales* presence protects from intestinal pathology, while a depletion of it, abolishes colonization resistance in the gut.¹³⁷ The bacterial family *Lachnospiraceae*, especially the genus *Clostridium*, has been identified in multiple studies as inversely correlated with inflammatory bowel disease.¹³⁸ A decrease in the abundance of *Lachnospiraceae* in the gut microbiota is associated with colorectal pathology.¹²⁷ Some members of the *Clostridiales* such as *R. gnavus*, an exclusive representative in breast-fed infants and segmented filamentous bacteria (SFB)

induce intestinal expression of Reg3γ, a critical antimicrobial peptide that maintains segregation between the epithelial layer and the microbiota.¹³⁸ Moreover, it has been suggested that *R. gnavus* ameliorate growth abnormalities and may help prevent malnutrition.¹³⁹ These findings are coherent with some major roles of VA. As an anti-inflammatory agent, VA has a protective influence on the gut barrier. By interacting with the immune system, it enhances the production of Reg3γ in the colon¹⁴⁰ and has an attenuating effect on colitis.¹ In case of VAD, it has been shown that SFB is reduced in the gut microbiome.¹⁴¹ On top of that, VA is a prominent factor to promote proper growth and to prevent malnutrition¹.

Lactobacillales

We know of a connection between certain lactic acid bacteria and VA. On the immune aspect, modulation of serum Immunoglobulin A production by the member *L. reuteri* is dependent on VA.¹⁴² In addition, it has been shown that Carotenoids, VA precursors, provide important biological functions for these bacteria, and several genes that involved in the production of carotenoids were determined in numerous lactic acid bacteria, such as *L. plantarum*.¹⁴³

We were interested to match our findings to a recent study with the closest similarity we were able to find to ours. This study compares the microbiome of VAS vs. VAD children with persistent diarrhea. Among their results, there were some resemblance to ours, e.g. the prevailing bacteria in the VAS group were class *Clostridia*, genus *Clostridium* and genus *Lactobacillus*. However, order *Lactobacillales*, was highly enriched in the VAD samples compared with the VAS ones, opposed to what we described.¹⁴⁴

5.4.4 PCV and the Microbiome

PCV was a major immune metadata characteristic that yielded the largest set of results in our study. We discovered significant differences in phylogenetic between and within distances, and specific bacteria of interest that were more represented in the PCV group: *Barnesiellaceae*, *Bacteroides*, *Lactobacillus agilis* and *Blautia producta*.

Streptococcus pneumoniae is a predominant cause of pneumonia, meningitis, and bacteremia. It is a leading killer of children under 5 years of age, responsible for the deaths of up to 2 million children annually, mainly in African and Asian developing countries. PCV7 protects against 50% of serotypes causing the disease in those high risk countries¹⁴⁵. Importantly, lung immunity is affected by the intestinal microbiome,

which induces Th1 and IgA responses via specific inflammasomes¹⁴⁶. There are studies that have already analyzed the nasopharyngeal microbial shift in PCV-vaccinated infants. A randomized controlled trial in the Netherlands of 97 PCV-7-vaccinated infants and 103 control infants found that PCV-7 immunization resulted in a temporary shift in microbial community composition and increased bacterial diversity. Furthermore, the abundance of *Haemophilus* and *Staphylococcus* bacteria in vaccinated was increased over that in controls¹⁴⁷. Another study showed that PCV7 shifts nasopharyngeal colonization and induces a temporary increase in *S. aureus* nasopharyngeal colonization in children around 12 months of age after the vaccine.¹⁴⁸ Studies of the relationship between the microbiome and the development and function of the immune system are demonstrating novel concepts. Several works show the impact of vaccines, particularly PCV, on the nasopharyngeal microbiome. Nasopharyngeal microbiota of infants undergoes significant changes after exposure to PCV7, which is mainly attributable to reduced prevalence of commensal bacterial families.¹⁴⁹

Barnesiellaceae

Three *Barnesiella* OTUs, bacterium that contributes to microbiota homeostasis, were significantly more abundant in the upper respiratory tract microbiota of mice before nasal inoculation with *S. pneumoniae*¹⁵⁰.

Bacteroides

Bacterial organisms from the *Bacteroidaceae* family are associated with pediatric vaccinations¹⁵¹. A study on influenza vaccination analyzed nasal bacterial composition in healthy adults at baseline and at 1 to 2 weeks and 4 to 6 weeks following the vaccine or intranasal sterile saline. The investigators found that the vaccine led to significant changes in nasal microbial community structure, diversity, and core taxonomic membership as well as increases in the relative abundances of *Bacteroides* genera¹⁵². Several studies showed that mainly *Bacteroides ovatus* was associated with immunization^{153,154}.

Here we present an innovative connection of PCV and the gut microbiome. The gut microbiota enhances primary alveolar macrophage function. Recent study identified a gut-lung axis during Infection and established a mechanism for pulmonary immunomodulation by the intestinal microbiota.¹⁵⁵ The intestinal microbiota plays a protective role during pneumococcal pneumonia.

Lactobacillus agilis

While lactic acid bacteria are generally non-motile, some of them are flagellated and exhibit motility. *Lactobacillus agilis* is representative of those flagellated microbes which reside in the gastrointestinal mucosa¹⁵⁶. There is evidence of *Lactobacillus agilis* isolation from stool samples of children suffering from kwashiorkor¹⁵⁷. Although we did not encounter a connection between *Lactobacillus agilis* and PCV, some other *Lactobacillus* species have an interaction with it. *Lactobacillus rhamnosus* administration for the prevention of pneumonia underscores the therapeutic potential of targeting the gut microbiota in pneumonia-derived sepsis¹⁵⁵. Also, Pregnant mothers who received *Lactobacillus rhamnosus* as probiotics every day from week 36 of gestation until delivery experienced reduced antibody levels specific for PCV7 than in the placebo group¹⁵⁸.

Blautia producta

The common feature of *Blautia spp.* is the utilization of hydrogen and carbon dioxide to form acetate. *Blautia spp.* are among the most abundant members of the entire gastrointestinal tract¹²⁷. Studies demonstrate that *Blautia producta* restores colonization resistance against pathogens in the intestines of mice, yet no reference to PCV.¹⁵⁹

Veillonellaceae

The relative abundance of the family *Veillonellaceae*, which is associated with milk polysaccharide digestion was significantly higher in breast-fed infants compared to the formula-fed¹⁶⁰.

Pseudoramibacter Eubacterium

The genus *Eubacterium* was for a long time recognized as one of the most abundant genera of the human gastrointestinal microbiota¹²⁷.

Yet, we have not found direct connection of the two latter OTUs to vaccination.

5.4.5 BW and the Microbiome

We revealed several bacteria of interest that were more represented in the NBW group: *Coriobacteriaceae* and *Blautia*.

Studies showed a significantly lower microbial diversity in LBW than NBW neonates. There were significant variations in the composition of placenta microbiota between the LBW and NBW neonates at the phylum and genus level¹⁶¹. In infancy, LBW mice have a more dysbiotic gut microbiome compared to NBW mice¹⁶².

Coriobacteriaceae

In light of the dominance and prevalence of *Coriobacteriaceae* in the mammalian gut, the literature speaks in favor of *Coriobacteriaceae* as being particularly relevant for host metabolic processes¹⁶³. A high abundance of *Coriobacteriaceae* could serve as markers of impaired maternal energy metabolism. Pregnant women with increased serum levels of insulin, an anabolic hormone, and elevated gestational weight gain, exhibit higher abundance of *Coriobacteriaceae*, especially *Collinsella*.¹⁶⁴ A study on African American women found that among women who delivered preterm, there was lower abundance of *Coriobacteriaceae*, compared with women delivering at term¹⁶⁵.

Unspecified species of *Blautia*

The taxa *Blautia* has been found to enriched with excessive gestational weight gain and maternal pre-pregnancy overweight or obesity^{166,167}.

Rothia mucilaginosa

Rothia mucilaginosa, and other *Rothia* species are part of the normal flora of the human oropharynx and upper respiratory tract. They are commonly associated with dental caries and periodontal disease, pneumonia and other infections, though no relation to prenatal growth was found¹⁶⁸.

5.4.6 Post-natal growth and the Microbiome

We found several relevant bacteria that are more represented in the retarded growth group: *Leuconostoc* and *Lachnospiraceae*.

Infant weight gain in the first year has been associated with the presence of different microbial groups¹⁰. Recent study showed that undernourished children in the Malawian birth cohort have immature gut microbiota that can cause impaired growth, and the transplant of growth discriminatory taxa is associated prevention of growth impairments¹³⁹. Furthermore, studies of children with undernutrition are highlighting the importance of postnatal development of the gut microbiota for achieving healthy growth and providing us with a new set of metrics to define the efficacy of nutritional recommendations and interventions directed at infants and the maternal-infant dyad¹⁶⁹.

***Leuconostoc* genus**

In a study about Malawian and Finnish infants, *Leuconostoc citreum* was the predominant species in both groups, but Malawian infants were more often colonized by this species¹⁷⁰. *Leuconostocaceae* family was associated with abnormal high

calprotectin levels, which is, a marker for intestinal inflammation that can affect growth¹⁷¹.

Lachnospiraceae, unspecified species of Blautia

Literature shows that members of *Lachnospiraceae* have been associated with protection against pathogen infections and obesity¹²⁸.

5.4.7 Gestational Malaria and the Microbiome

We revealed that mainly *Bacteroides* were represented in mothers with gestational malaria.

The gut microbiota of mice influences the pathogenesis of malaria. Mice which exhibited differences in their gut bacterial community had significant differences in parasite burden and mortality after infection with multiple *Plasmodium* species¹⁷².

Notably, strategic modulation of gut microbiota composition could decrease the risk of *P. falciparum* infection in malaria-endemic areas¹⁷³.

Bacteroides

Bacteroides species are significant clinical pathogens and may be passed from mother to child during vaginal birth and thus, become part of the human flora in the earliest stages of life^{174,175}. Children in a rural African village in Burkina Faso have an enrichment of the *Bacteroidetes* phylum compared with European children¹⁷⁶. Prior works show that *Bacteroids* strongly correlate with malaria infected mice.¹⁷⁷ Analysis of the cecal bacterial communities revealed *Bacteroidaceae* were proportionally more abundant in susceptible mice¹⁷².

6 Conclusions

Overall, we presented a complex set of correlations referring to infant's VASUP, VA status, growth and immune state, as well as mother's course of gestation, And the effect of these on the microbiome of the infant.

We found that VASUP slightly affects the microbiome of the recipients. The effect is mostly at the individual within level. We could not spot specific bacteria which change as a result of VASUP.

Interestingly, we revealed distinctions in the microbiome composition of children with different VA status.

Moreover, we discovered metadata growth and immune features such as: PCV, pre-natal and post-natal growth, gestational malaria, that influence the microbiome in various extents.

Furthermore, we found important correlations between VA status of children and their growth, immune state and maternal diet.

There is a major concern among physicians and nutritionists as to whether nutritional supplementation alters the composition of the microbiome. Here we have shown that high dosage VASUP has an effect, yet a subtle one, on the microbiome.

This project is innovative as no research has thoroughly evaluated to date the contribution of VASUP and other health and nutrition aspects on the gut microbiome of African infants.

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תקציר

מבוא

רמות הולמות של ויטמין A דרשות לתקינות יצירת האיברים, הגדילה, תפקוד מערכת החיסון, התמיינות הרקמות, פעילות מערכת העיכול והראיה.

ויטמין A תפקיד מكيف במערכת החיסון. במערכת החיסון המולדת, מחסור בויטמין A משפייע על שלמות המחסום המוקוזלי בmund ועל הפרשת אימונוגלובולין A, בעוד שבעוד שבערכת החיסון הנרכשת, מחסור בויטמין משפייע לשילוה על ייצור תא T, במיוחד Th17 ו-Treg FoxP3⁺.

מחסור בויטמין A משפייע על כ-250 מיליון ילדים בגלאי טרומ בית ספר, במיוחד במדינות מתפתחות, שם הוא מגדיל תחלואה ומות מזיהומיים חמורים. תוסוף במינון גבוה של ויטמין A נועד להעלות את מאגרי הויטמין בכבד ולאפשר שחרור הדרגתית והובלה של הויטמין לרകמות ילדים עם מחסורים תזונתיים של ויטמין A.

המיקروبויום הוא יישות נוספת שמקורת למערכת החיסון. ההשפעה של המיקروبויום היא הן על המערכת המולדת והן על הנרכשת, למשל: ישנה אינטראקציה בין המיקروبויום לבין תא T, תא B ותא מערכות החיסון של אפיתל המעי.

מיקרוביום המעי בינווקט מושפע מاؤגן הלידה, שבוע הלידה, תזונת התינוק, אתניות, עליה במשקל ופקטורים נוספים. מיקרוביום המעי של תינוקות שנולדו בזמן מרכיב בעיקר מ-4 מערכות: אקטינובקטריה, בקטרואידיטים, פירמיקוטים ופרוטואבקטריה.

ויטמין A יכול להשפיע על תగבות חיסוניות ישירות, על ידי אינטראקציה עם תא החיסון, או בעקיפין, על ידי אינטראקציה עם המיקروبויום. ראשית, מחסור בויטמין A יכול להוביל לדיסביזיס (חוסר איזון באוכלוויות החידקים) על ידי השפעה על השכיחות היחסית של חידקי מעי מסוימים. שנית, סיגנלים שקשורים ל-A₇ בתא B חיוניים להרכבת מיקروبויום תקין. שלישיית, סינרגיה בין ויטמין A לבין סיגנלים מיקרוביאליים מקדמת התמיינות של תא Th17. לבסוף, מחסור בויטמין A משפייע לשילוה על תהיליך הפוקוזילציה, שחינוי לתמיינה בחידקים הקומנסליים (אלו שחיים בסימביוזה עם בני האדם). לאחרונה, נחקר הקשר בין פרוביוטיקה ושיפור סטטוס של ויטמין A.

המיקروبויום, בתרו, יכול להשפיע על המטבוליזם של ויטמין A. גירוי של תאים דנדרייטים דרך סיגנלים מיקרוביאליים מעלה את הייצור של ויטמין A. מצד אחד, בפיזובקטריות אינפנטיס, שנדגם על ידי תא החיסון, מעלה את האנזימים האחראים על מטבוליזם של ויטמין

A, ומצד שני, האפקט האנטי-דלקתי של ביפידובקטריום אינפנטיס גם תלוי בנסיבות של ויטמין A.

ההיפותזה שלנו היא שתיסוף ויטמין A, שמשפר את הסטטוס האימונולוגי, ישנה גם בזורה משמעותית את הרכב המיקרוביום.

שיטות

מחקר השוואתי שבוסס על אוכלוסייה בוצע באוגנדה. 100 צמדים של ילדים בגילאי 6-24 חודשים והאימהות שלהם גויסו למחקר. בין הילדים שגויסו, היו ילדים עם מחסור בויטמין A וכאלו עם רמה מספקת של הויטמין.

איסוף נתונים מבוסס זמן התחיל בקן ההתחל, כלומר: לפני מתן תוסוף ויטמין A והמשיר ב- 3 נקודות זמן נוספות: יום לאחר התisisוף, שבוע לאחר התisisוף וחודש לאחר התisisוף.

מתוך 100 הילדים שגויסו למחקר, רק 67 הגיעו איסוף מלא (של כל 4 נקודות הזמן), ואלו שימושו לאנלייזת המשך.

שאלונים בנוגע למגדדים אנטרופומטריים, מידע חיסוני ותזונתי אודות צמדי אם-ילד מולאו על ידי האימהות.

דגימות דם להערכת רמות ויטמין A נאספו ב-2 נקודות זמן ונבדקו על ידי O₂ FLUORO Check*i* בשטח ועל ידי HPLC במעבדה.

דגימות צואה לאנלייזת במיקרוביום (rRNA 16S) נאספו ב-4 נקודות זמן והאנלייזה כללה: בידוד DNA, PCR וטיהור תוצריו, כימות וחיבור התוצרים יחד על מנת לבצע ריצוף בעזרת Illumina MiSeq.

אנליזת רצף הגן rRNA 16S בוצעה במספר כלים ביואינפורטטיבים ובווטטיטיבים: QIIME, LefSe, MaAsLin, PICRUSt

תוצאות

אוכלוסיות החידקים של התינוקות במחקר שלנו כוללה 4 מערכות עיקריות: אקטינובקטריה (48.6%), פירמיקוטים (27.1%), פרוטיאובקטריה (16.3%) ובקטריאידיטים (7.4%). הסוג ביפידובקטריום הרכיב כמעט את כל האקטינובקטריה (47%). חידקים עיקריים נוספים היו סדרת קלוסטרידיאלים (18%), משפחת אנטרוביוטריאוצה (15.7%), סוג *Willowella* (13.7%), סדרת לקטובצילאלס (9.1%), סוג *Streptococcus* (6.2%) ומשפחת פרוטוליצה (4%).

כשבחנו את המרחקים התור-אינדיידואלים של המיקרוביום, מצאנו הבדל מובהק במרחקים המיקרובייאליים לפני תיסוף ויטמין A ויום לאחר התיסוף ($p=0.0009$, FDR), ובנוסף, לפני התיסוף ושבוע לאחר התיסוף ($p=0.008$, FDR). יתרה מזו, מצאנו מובاهקות כאשר השוינו מרחוקים של יום אחריו התיסוף לבין חדש לאחר התיסוף ($p=0.02$, FDR). לא נמצאו שינויי מובהקים בחידקים מסוימים כתוצאה מההתיסוף.

לא תלות בתיסוף ויטמין A, נצפה שינוי בחידקים מסוימים לפני סטטוס A של התינוקות בהתחלה的研究: מחלוקת בטאפרוטאובקטריה, סדרת קלוסטרידיאליים, משפחת *լכטוספירה*, סדרת *לקטובצללים*.

המאפיין העיקרי שהופיע על הרכב המיקרוביום היה חיסון VCV. גילינו הן שונות אלו (שינויים בתוך הפרט) והן שונות בטא (שינויים בתוך הקבוצה) בין הילדים שחוינו לבין אלו שלא. מצאנו 21 חידקים שהיו מייצגים יותר בקבוצת הילדים שחוינו.

גדילה תוך רחמית, גדילה בחצי השנה הראשונה לחיים ומילריה בהריון השפיעו גם הם על חידקים ספציפיים בתינוקות: *קווריבקטריצה* ובלאותיה; *לאוקונוסטוק* ולכטוספירה; ובקטרואידס, בהתאם.

לא פחות חשוב מכך, בחנו קשרים בין ויטמין A, והיבטים של גדילה, מערכת החיסון ותזונה. מצאנו כי סטטוס תקין של ויטמין A מקשר עם גדילה טובת יותר לאחר הלידה. קבלת אנטיבiotיקה וחיסון VCV מקושרים לרמה גבוהה יותר של ויטמין A אצל התינוקות. גילינו קורולציה בין תזונת האם וסטטוס ויטמין A של התינוק.

כנאליזת משנה מצאנו אינטראקציות שונות בהיבטי גדילה וחיסוניות התינוק, כמו כן, בהיבטי בריאות ותזונת האם.

דיון

הממצאים שלנו על אוכלוסיית המיקרוביום במחקר תואמים את המחקרים העדכניים על מיקרוביום תינוקות, עם חידקים מסוימים שהם חלק בלתי נפרד ממנו.

הינו עדים להשפעה עדינה של תיסוף ויטמין A על המיקרוביום ברמה אינדיידואלית. מחקרים קודמים גם תיארו שונות קטנה בין אוכלוסיות לעומת שונות בתוך האוכלוסיות. השינוי במיקרוביום במהלך שבוע, ובתוך במהלך יום צריך להיות מוסבר על ידי אפקט התיסוף. מצאנו גורמים נוספים שהשפעו על המיקרוביום, וכך היה זה מתאים להסיק מסקנות על האפקט הייחודי של התיסוף. בנוסף, אנחנו מאמינים שהנחה בילדית, פקטו ר המשפיע רבות על המיקרוביום בינקות, הייתה יכולה למסור על השפעת התיסוף על המיקרוביום.

באופן מעניין, גילינו סטטוס ויטמין A של התינוקות בהתחלה המחבר משפיע על הרכב המיקרוביום עם חידקים מסוימים שכחחים יותר בקבוצת הילדים עם רמה מסוימת של A. ידוע לנו על קשר בין כמה חידקים שמצאו כmo: *לקטובצילאלוס*, *קלוסטרידיאלוס*, *לכנופירצה לבין* ויטמין A.

סטרפטוקוקוז פניאומוניה הוא הגורם הבולט לדלקת ריאות והוא הסיבה המובילה להרג של ילדים מתחת לגיל 5, בעיקר במדינות מפותחות. חיסון PCV מגן בפני חצי מהסרוטיפים השגורמים למחלה בארצות אלו. ישנו מחקרים שכבר מצאו שינוי במיקרוביום דרכי הנשימה בתינוקות שחווו עם PCV. כאן אנו מראים קשר חדש בין PCV ומיקרוביום המעי. גילינו שינויים מובהקים במרקחים פילוגנטיים בתוך ובין הפרטיטים, וחידקים ספציפיים שייתר שכחחים בקבוצת התינוקות שחווו: *ברנס'אלצה*, *בקטרואידס*, *לקטובצילואג'לאס* ובלואטיה פרודוקטה.

יתרה מזו, מצאנו קורולציה בין גדיית התינוק ומחלות בהריון לחידקים שרלוונטיים בספרות למאפיינים אלו.

כדי לחזק קביעות קודמות ולהוסיף עליה, הראיינו כי סטטוס ויטמין A מקשר לגדיית התינוק, שימוש באנטיבiotיקה, חיסון PCV ותזונת האם.

מסקנות

בכללי, הצגנו סט מרכיב של קורולציות המתיחס לתיסוף ויטמין A, סטטוס ויטמין A, גדייה סטטוס חיסוני ומהלך ההריון, והשפעה של אלו על מיקרוביום התינוק.

מצאנו שתיסוף ויטמין A בעל השפעה לא גדולה על המיקרוביום של התינוקות. האפקט הוא בעיקר ברמת האינדיבידואל. לא מצאנו חידקים מסוימים שמשתנים כתוצאה מהתיסוף.

באופן מעניין, מצאנו הבדלים בהרכב המיקרוביום ילדים עם סטטוס ויטמין A שונה.

יתרה מכך, מצאנו מאפייני גדייה ומערכות חיסון כמו: PCV, גדייה תוך רחמית, גדייה בחצי שנה הראשונה לחיים ומלהריה בהריון, שהשפעו על המיקרוביום בהיקפים שונים.

ישנו עניין גדול בקרב רופאים ותזונאים בנוגע ליכולת של תוסף תזונתי לשנות את הרכב המיקרוביום. כאן הראיינו שתיסוף ויטמין A במינון גבוה משפיע, אולם בצורה קטנה, על המיקרוביום.

האפקט של תיסוף ויטמין A, סטטוו ויטמין A והיבטים חיסוניים על המיקרוביום של תינוקות

עבודת גמר

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РХОВОТО, ДЦМБР 2018