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Characterization of the infant gut microbiota in a cohort of 330 Danish children from 9, 18 and 36 months by quantitative PCR array (GULDA) analysis

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Introduction
We have developed a qPCR-based array (GUT Low Density Array, GULDA), which simultaneously determine the relative abundance of >30 different bacterial 16S rRNA gene targets in a given DNA-sample covering selected phylogenetic levels (Bergström et al., FEMS Microbiology Letters 337 (1), 36-47, 2012). GULDA was applied to fecal DNA from 330 healthy Danish infants, sampled at 9, 18 and 36 months after birth, enabling characterization of interindividual relationships by multivariate data analysis (Principal Component Analysis, univariate data analysis (ANOVA, t-tests) and non-parametric pairwise Spearman correlations. Interpretation of these patterns in relation to previously determined nutritional, anthropometrical (growth indices), and blood sampled parameters was used to increase understanding of gut microbial physiology. Particular emphasis will be given to possible early life microbiota biomarkers of obesity, given the correlation of early life overweight with adult obesity and related lifestyle diseases. Few studies have undertaken similar longitudinal and multiparametric analysis for such numerous participants. The concomitant measures on pressure from all relevant phyla on multiple taxonomic levels give a unique possibility for recognition of gut bacteria clusters. Due to study dropout, non-compliance and failed fecal DNA purifications, at total of 658 fecal samples were available for the analysis (>200 in each of the three age groups).

With exception of breastfeeding and certain obesity indices (Figures 6 and 7), we found quite few consistent correlations between the gut bacteria and the physiological parameters, hence the primary focus of the current presentation is on the interbacterial correlations.

Figure 1 – Principal component analysis (PCA) of the GULDA microbiota. Upper plot-Individuals (Scores): Lower plot-Bacteria (Loading). This figure shows the two primary principal components, PC1 and PC2, which explain approximately 30% of data variation. Only individuals who completed all three fecal samplings were included, giving a total of 396 samples from 132 individuals. There is a strong temporal development moving along PC1 from left to right, moving from 9 to 36 months, and a moderate temporal development moving along PC2, from top and down. Higher diversity of 9 months samples with corresponding fewer bacterial species relative to 18 and 36 months is evident. Bacteria codes (see Figure 3).

Figure 2: Temporal development of the gut microbial composition isoG (Phylogenetic) of all GULDA bacteria from 9 to 18 months, 18 to 36 months, and 9 to 36 months. Overall the majority of changes in the gut composition take place between 9 and 18 months with less change occurring between 18 and 36 months. Specific increases were seen for most of the targeted Bacteroides, C. leptum, E. hallii, Roseburia spp., Enterococcus spp., Lactobacillus spp., and in Lactobacillus salivarius. A few GULDA targets showed opposing changes compared to the temporal development of the respective phylogenetic target. Statistical significance of one-sided t-tests.* p<0.05, ** p<0.01, *** p<0.001

Figure 3: Spearman pairwise correlation map of all interindividual correlations at 9, 18, and 36 months sampling. Not surprisingly, many positive correlations of targets belonging to the same phylum were observed. At 9 months most members of the Bacteroidetes phyllum (B1-B6, B8) were negatively correlated to the Firmicutes phyllum, including Enterococcus spp., however such correlations were not observed at either 18 nor 36 months. Indeed at both 18 and 36 months significant positive correlations were found between several bacterial groups within the Firmicutes, including C. butyricum, the C. leptum group and the C. cocoides group and members of the Bacteroidetes including Bacteroides spp. and Alistipes spp. This development may reflect dietary changes as breastfeeding is weaned off at each of the three adult diet, rich in long-chain carbohydrates and animal fats and protein, is introduced between 9 and 18 months. Interestingly, Prevotella spp. showed negative correlation to specific Bacteroidetes targets at 36 months, possibly indicating the earliest observed signs of enterotype stratification. Since each time point was analyzed separately, the included number of individuals was >200 in each group.

Figure 4: Enterotype development from 9 to 36 months. Relative abundances of Bacteroidetes and Prevotella targets show a distinct development from 9 to 36 months (A-C), arguably moving from trace amounts of either bacteria just after birth (blue circle) to a Bacteroidetes dominated microbiota (red circle) at 9 months. From 9 to 36 months, an increasing, yet smaller subgroup of Prevotella dominated individuals appear (green circle), indicating segregation of specific individuals from a Bacteroidetes dominated into a Prevotella dominated enterotype. These findings were paralleled by the temporal development of frequency distributions of Bacteroides (F1-F5) and Prevotella (G6) and particularly of the P/B-ratio from 9 to 36 months. A distinct bimodal P/B-pattern seems to be established from 9 to 36 months (L-L), clearly driven by stratification into individuals with either high (green) or low (red) relative Prevotella abundances. Possible causes of this segregation could be genetic or dietary, but could not be clarified in the present study. Since valid qPCR results for both primer sets were required at all time points, the number of individuals was lower in than presented.

Figure 5: Enterotype shifts from 18 to 36 months Examination of individuals giving valid qPCR results for both 18 and 36 months samplings, illustrated that: 48/79 were in the low P/B group, while 8/79 were in the high P/B at both time point 14/79 and 9/79 shifted enterotype from low to high and high to low P/B, respectively. The separation into the two enterotypes is quite clear. Comparison of individuals shifting to the high P/B group with individuals staying in the lower P/B group showed a slight significant correlation to increase in BMI, but these results should be taken with precautions, given the large differences in group sizes.

Figure 6: Effect of breastfeeding on infant gut microbiota Numbers denote p value of Mann-Whitney statistical test between the relative abundances of the GULDA bacteria at 9, 18 and 36 months, dependent on whether or not, the infants were still breastfed at the 9 months examination. Cessation of breastfeeding seems to be the major factor in maturating the gut microbiota, but the effect wanes off for most targets after 9 months.

Conclusions
We found significant developments in the gut microbiota from 9 to 18 months, where cessation of breastfeeding and introduction of a Westernized diet induces replacement of a simpler, less diverse lactobacilli and bifidobacteria dominated microbiota with larger Clostridia (with polysaccharide preference) and Bacteroides (with animal fats, protein preference) targets. Moreover, we report the earliest signs of enterotype segregation as the development of microbiota characterized by either high or low relative levels of Bacteroidetes/Prevotella, seems to take place between 9 and 36 months. Correlations between zBMI and specific Clostridia from 9 to 18 months and zBMI and a tendency to a shift to the Prevotella-rich enterotype from 18 to 36 months may indicate specific carbohydrates to be of interest in relation to obesity development.