

RESEARCH PAPER

Microarray analyses of the infant gut microbiota

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ABSTRACT:

To detect the cause of germ free infant gut become colonization after birth and develops though the pregnancy for mother, 4 days, 10 days, 4 months, 1 year to 2 years is important, which has strong relationship with health and disease infant. This research was carried out at the experimental laboratory of molecular microbiology department at Norwegian University Of Life Science, College of of NMBU, Ås la Norge / Norway, during Jan 2015 to get more information about the development of microbiota in infant. The pattern of colonizers is different at the different age. The purify faecal sample DNA perform universal amplification of the 16Sr RNA to the determination of gut microbiota by the GA-map infant array and 454 pyrosequencing. The results showed implicating the *Bifidobacteria* and *Firmicutes* that present in great amount during the development of the infant gut microbiota with reach to peak at the age of several weeks. A temporal trend in the microbiota composition was also detected which indicates the usefulness of this method for determining the temporal dynamics of the infant gut development.

KEY WORDS:

Microbiota ,Gut, Fecal,Infant.

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INTRODUCTION :

The human gastrointestinal tract is densely populated by a large amount microbial ecosystem. One adult human whole organ bears generally more microbial cells than body cells whereas only our intestinal tract contains 100 trillion microbes and the vast majority microbes (10^{11} - 10^{12}) have found in our colon (Whitman *et al.*, 1998). Though there is a

universal relationship between the host and its gut microbiota, vast majority is still now unknown. The microbial communities inhabiting our gut offer evolutionary conserved services like synthesizing essential molecules, strengthening our immune system, enhancing nutrient uptake, stimulating angiogenesis, and regulating host fat storage (Marzorati *et al.*, 2011; Palmer *et al.*, 2007 and Woodmansey, 2007).

Unfortunately, some of these organisms are opportunistic pathogens, and many can cause harm if the normal and healthy community composition in the host is altered (Round and Mazmanian, 2009 and Woodmansey, 2007). The development of the human infant gut microbiota is yet known little which is mainly due to methodological constraints. The workload associated generating and analyzing microbiota data and the complexity are the current challenges.

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Children with antibiotic treatment and caesarean delivery represent major perturbations of the microbiota (Marques *et al.*, 2010 and Rudi *et al.*, 2007a). Early colonizers are staphylococci and streptococci whereas clostridia and Bifidobacteria increase with age (Trosvik *et al.*, 2009). The pattern of early colonization is important because this leads the immune system not respond properly later in life (Kalliomaki *et al.*, 2001). Therefore, an understanding of the gut microbiota development in infants has the possibility to develop strategies to modify the microbiota for the current epidemics in allergic disorder development in the western world. The change of lifestyle or use of antibiotic can thus strong affect the prevalence of selected gastrointestinal microbes playing an essential role in normal gut development and health (Woodmansey, 2007). Some time it can cause diseases like intestinal cancer, necrotizing enterocolitis and inflammatory bowel disease in infants (Palmer *et al.*, 2007; Round and Mazmanian, 2009 and Sekelja *et al.*, 2011). Any change in the optimal environment in the gut, due to change in ratio between microbis, and this linked to increase diseases such as asthma, allergic disorders and obesity (Blaser and Falkow, 2009; Flint, 2011; Kalliomaki *et al.*, 2001; Nakayama *et al.*, 2011; Round and Mazmanian, 2009 and Sekelja *et al.*, 2011).

The characteristics of the gene encoding the 16S ribosomal RNA (Woese, 1987), is useful for classification of both higher taxa and closely related species, and used in combination with different molecular techniques to analyze bacterial diversity (Blaser and Falkow, 2009; Momozawa *et al.*, 2011; Nakayama *et al.*, 2011; Rudi *et al.*, 2007b and Zwielehner *et al.*, 2009). Cloning and DNA sequencing is used for getting specific information out of unknown samples. Probe-based methods are generally used if the bacteria searched for are already known. Direct sequencing and electrophoreses-based methods such as DGGE, TGGE and T-RFLP are used for screening purposes and pattern recognition (Rudi, 2013).

Stool samples are very easy for study and analyzing bacterial diversity in the gut, but stool samples are not contain all a microbiota composition reflecting the one existing in the whole colon or small intestine (Marchesi, 2011), a stool sample only reflects the composition of

microbiota present in the lumen, not of that growing on the mucosal surface (Momozawa *et al.*, 2011 and Pedersen and Tannock, 1989). The studies of functional biofilms in the gastrointestinal tract are preferable to alternative approaches, but they are limited by the inaccessibility to most of the areas that together make up the digestive tract (Marchesi, 2011).

The distal gut has much attention by researchers because of it high microbial density (Whitman *et al.*, 1998), and easy accessibility. There are some claim that infant is not sterile at birth after all, because DNA from *Bifidobacterium* and *Lactobacillus* has been detected in the infant placenta (Satokari *et al.*, 2009).

Some bacterial genera were detected in the meconium from 21 healthy newborns as recorded by (Jimenez *et al.*, 2008).

The relationship between human and gut microbiota is complex, and there is increasing in developing microbiological interventions as a strategy for preventing health problems and disease, more studies, more improvement of techniques is highly necessary to be able to answer. Lab experimental a 16S rRNA gene microarray approach was used to determine the development of the gut microbiota in a cohort of mothers and their infants from 4 days to 2 year of age. The rationale was that the temporal development of the infant gut microbiota is an important parameter for the health of the infant, and challenges with probe specificity and cross-reactivity between closely related species was met by using highly specific single nucleotide primer extension (SNUPE) probes. The aim of this research to get more information about the development of microbiota in infant, results obtained by this study can give ideas of what has to be improved in future research.

2. MATERIALS AND METHODS

Research was carried out at the experimental laboratory of molecular microbiology department at Norwegian University Of Life Science, College of of NMBU, Ås la Norge / Norway, during Jan 2015 to get more information about the development of microbiota in infant. Each group was processed four fecal samples from a

mother/child, in addition to a positive and a negative DNA purification control (8 samples in total). The infant fecal samples were collected during pregnancy for the mother, and at the age of 4 days, 10 days, 4 months, 1 year and 2 years. The feces have been frozen immediately at -80°C to prevent degradation and bacterial growth.

The samples got as follows:

1. Late pregnant sample
2. 3 days sample
3. 10 days sample
4. 1 year sample
5. A positive control
6. A negative control

2.1. DNA isolation from stool samples:

To extract DNA from all species in all samples, bacterial cells in all samples were lysed by mechanical disruption using glass beads. For this, 40 mg stool sample was dissolved in 1200 µl binding buffer and 0.25 g of <106 µm glass beads. They were vortexed briefly and frozen down at -20°C. The cells were homogenized in the MagNA Lyser (6500 rpm in 2×20 sec) and it was kept on ice between each homogenisation step. It was then centrifuged at 13000 rpm for 5 min at RT and the supernatant was transferred to a new microcentrifuge tube. 10 µl SiMAG/ MP-DNA Magnetic beads (200 mg/ml) were added and the sample (400 µl) was transferred with beads to the designated wells in the Sample plate. 1 ml of washing buffer 1 was aliquoted into the designated wells in Wash plate 1 and 1 ml of washing buffer II designated wells in Wash plate 2 and 1 ml of washing buffer III designated wells in Wash plate 3. Finally, 100 µl elution buffer was aliquoted into the Elution plate and then ThermoScientific King Fisher® Flex robot was switched on. After the extraction was finished, the isolated DNA was in Elution plate (Rudi, 2013).

2.2. Quantification of genomic DNA.

For quantification of genomic DNA we used Quant-It Assay for Qbit.

2.3. PCR amplification by primers targeting universally conserved regions of the 16S rRNA gene:

A new universal PCR, Cover All PCR amplifying an approximately 1200 pb region of the 16S rRNA gene was used for the amplification of universally conserved region of the 16S rRNA gene all six samples in addition with positive control (*E. coli* DNA) and negative control (water). After PCR amplification, to verify the quality of the PCR product we run it in Agarose gel electrophoresis and used 1 Kb ladder (N3232) for quantification. After visualized the bands from gel in UV light (Rudi, 2013).

2.4. PCR product quantification (picogreen reagent)

The PicoGreen fluorescence quantification was done with the FLX 800cse instrument which measures the PicoGreen signal that evolves when the fluorochrome is bound to dsDNA (Rudi, 2013). This gives a specific measurement of the DNA present in the solution.

2.5. Array analyses

All samples from each group of the ExoSAP-treated PCR-products were labeled after the SNUPE principle, according to the protocol (Rudi, 2013), depending on which probe set used, different bacteria can be detected with the microarray method. The probe designing strategy used for the GA- Map™ infant assay.

The GA-Map array method was based on the use of SNUPE labeled probes there are many probes are labeled in the same reaction. The SNUPE probes were made so that the probes hybridized adjacent to identified gene location if the target bacterium was found then a labeled dideoxynucleotide was incorporated by the polymerase (Fig 1, 2).

The same protocol as for measurement of genomic DNA was used for PCR product quantification.

In array analysis, 4 steps were followed as given below:

1. ExoI-SAP treatment for removing residual PCR primers and dephosphorylation inactivation of nucleotides used in PCR.
 2. Probe endlabeling by SNUPE.
 3. Hybridization of the labeled probes to their respective complementary oligonucleotides spotted on an array.
 4. Scanning and analysis of hybridized array.
- The Exo-Sap treatment was done to make master mix for 15 µl PCR product and

added to the PCR wells. Then it was incubated on a thermal cycler. Then 8 µl of the master mixture was added to the wells. 2 µl template was added to the respective wells. 10 µl of this reaction was preceded for PCR (Rudi, 2013).

2.6. Array analyses by MAGPIX

The MAGPIX system is a versatile multiplexing platform capable of performing qualitative and quantitative analysis of proteins and nucleic acids in a variety of sample matrices. This affordable system requires less sample input than many other current technologies and can perform up to 50 tests in a single reaction volume, greatly reducing sample input, reagents and labour while improving productivity (Rudi, 2013).

2.7. Pyrosequencing

In the [DNA sequencing](#) depended on the [pyrophosphate](#) release when nucleotide incorporation by activity of [DNA polymerase](#) “Sequencing by synthesis”. This method is based on identification of the activity of [DNA polymerase](#) after amplification all samples were mixed in a tube purified on column and then sending to the 454GSFLX instrument at the Norwegian High-Throughput Sequencing Centre (Rudi, 2013).

2.8. Purification of PCR product using E.Z.N.A. DNA purification kit

Added 250 µl of Buffer CP to 50 µl of mixed PCR product in a 1.5 ml tube, and vortexed thoroughly to mix; spined the tube to collect the drop from the inside of the lid. Then applied the sample to the HiBind® DNA column with 2 ml collection tube and centrifuge at 15000 rpm for 1 min at (RT). After that centrifugation we washed the HiBind® DNA column with 700 µl of DNA Wash Buffer and centrifuge at 15000 rpm for 1 min at RT. Liquid discarded in the from 2 ml collection tube and repeated previous

centrifugation step using 500 µl of DNA Wash Buffer. Similarly discarded the liquid and centrifuge the empty HiBind® DNA column for per min at 15000 rpm. Then placed HiBind® DNA column into a clean 1.5 ml tube, added 30 µl of Elution Buffer (10 mM Tris, pH 8.5) onto the column matrix and centrifuge for 1 min at 15000 rpm to elute DNA. Then transferred eluted Elution Buffer with DNA from 1.5 ml back on the column, centrifuged as in previous step once again. The step is measurement of the the eluted DNA was performed by the Qubit Quant It assay (Rudi, 2013).

2.9. Probe Verification by Quantitive PCR

Quantitive PCR was done to enable accurate quantification of specific DNA targets.

For this two qPCR reactions were set up for each DNA sample. In one reaction, universal 16S rRNA gene primers and probes, and the reaction were with *Bifidobacterium longum* primers and probes.

3. RESULT AND DISCUSSION

The study began with the purification of faecal DNA. For these 8 samples were preceded with a positive control and negative control. The positive control was to determine the technical errors in the DNA purification, while the negative control was to check potential contamination. All samples were processed for DNA isolation and quantification of genomic DNA was performed by using both PicoGreen reagent and Qbit measurement. As picogreen is light sensitive. The PicoGreen measurements of the genomic DNA from the stool samples showed a successful extraction and the extraction gave good parallel results (Fig, 1).

The objective of this experiment is to determination of the development of the microbiota infant gut of human and to find out the correlation between health and disease by detection and understand the diversity of microbiota. For these 6 samples were preceded with a positive control and negative control. The positive control was to determine the technical errors in the DNA purification, while the negative control was to check potential contamination. All

samples were processed for DNA isolation and quantification of genomic DNA was performed by using PicoGreen reagent and Qubit measure. In current experimental DNA extraction in

combination with the use of Cover All primers was used (Fig, 11).

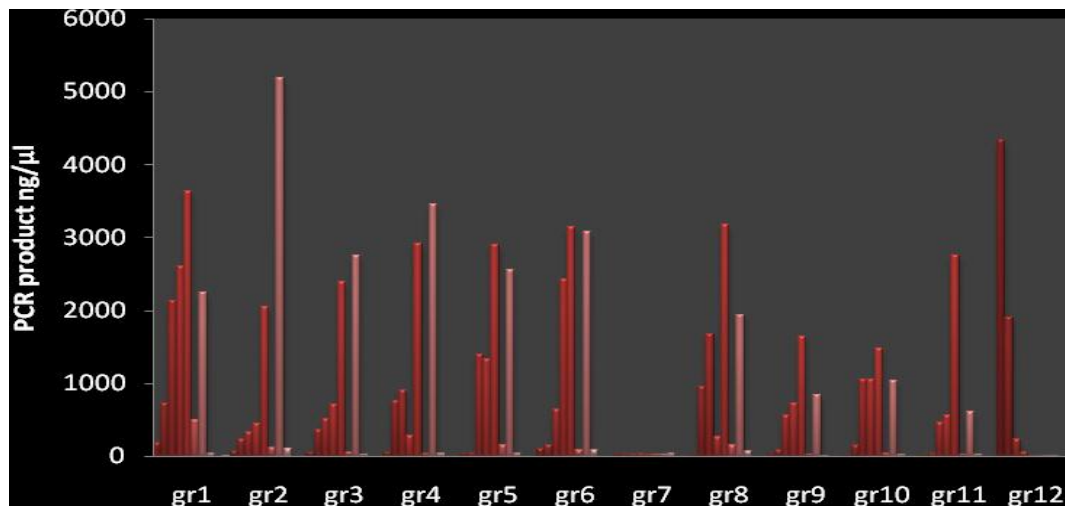


Fig 1: Amount of amplified DNA (ng/μl) from the Coverall 16S rRNA PCR, quantified by using PicoGreen.

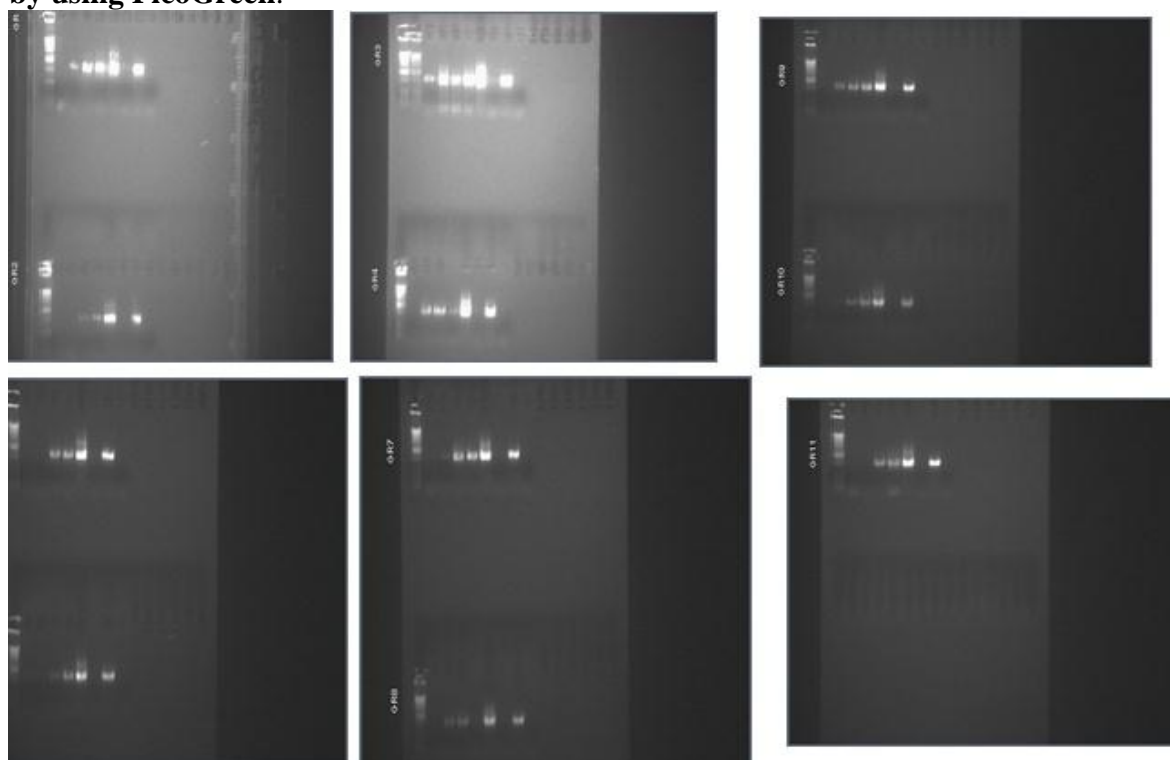


Fig 2: Agarose gel results of all samples showing the 16S rRNA bands and thus a successful. Amplification of genomic DNA in all samples(PCR amplification was performed using primer pairs GC-ZF and 1622R coupled with a GC-tail and run on DGGE to determine the banding pattern specific to each sample).

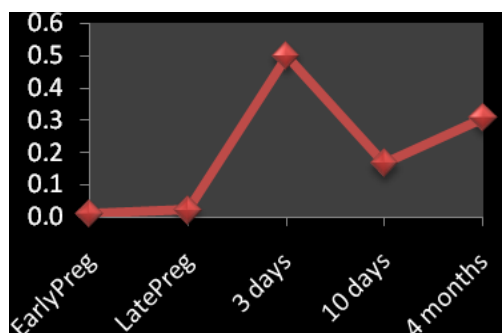


Fig3: Correlation between age (gr.2411) and Microarray *B.no longum*

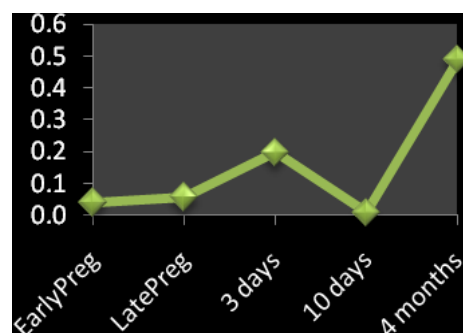


Fig4: Correlation between age (gr.2411) and Microarray *B.longum*

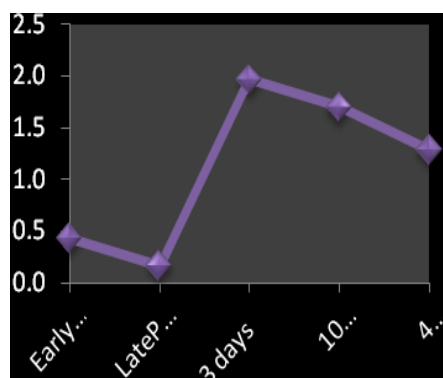


Fig5: Correlation between age (gr.2411) and pyrosequencing qPCR *B.longum*

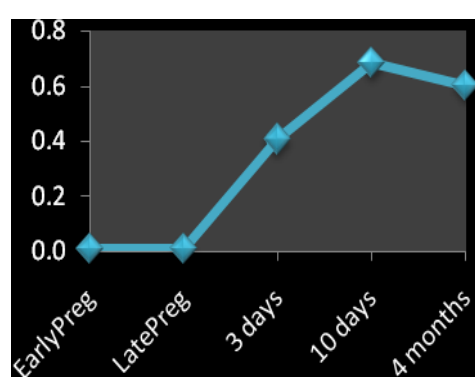


Fig6: Correlation between age (gr.2411) and *B.longum*/Total.

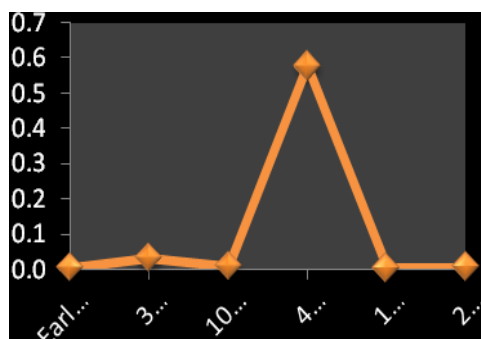


Fig7: Correlation between age (gr.2206) and microarray (gr.2206) and microarray *B.longum*

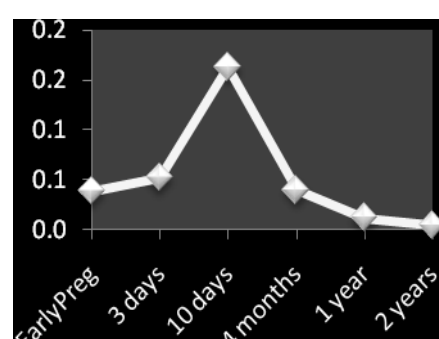


Fig8: Correlation between age *B.nolongum*.

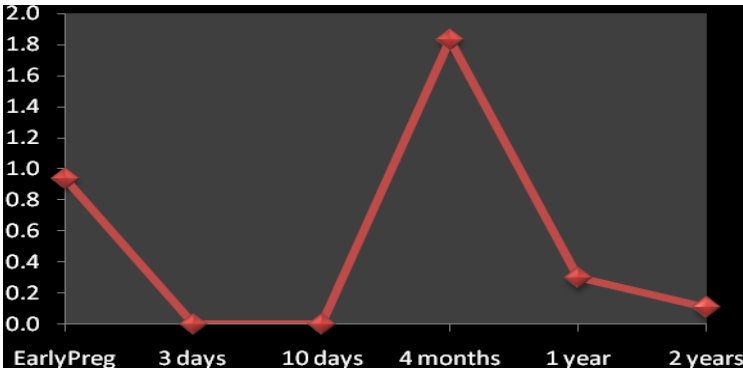


Fig9: Correlation between age (gr.2206) and qPCR *B. longum*.

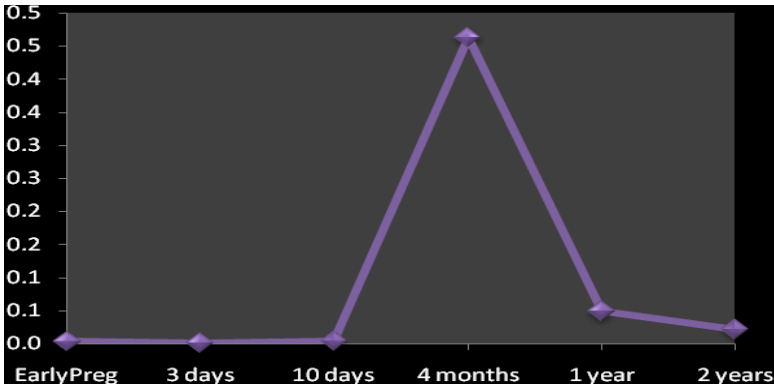


Fig10: Correlation between age (gr.2206) and pyrosequencing *B. longum*/Total.

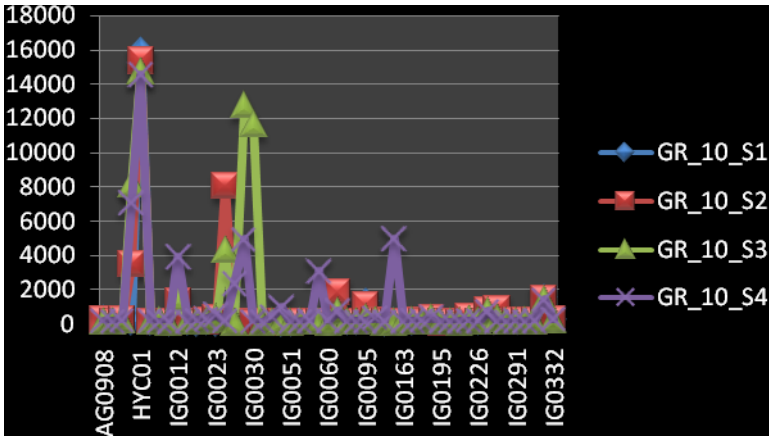


Fig 11: GA-map array the probes of bacteria in the sample of groups 10.

Table 1: List of target groups for Probe set NEC v. 1.0 showing different types of probes in the different stage of age.

Probe #	Bacteria
UNI05	Universal
IG0005	Proteobacteria
IG0178	Gamma-proteo group
IG0056	Gamma-proteobacteria subgroup
IG0011	Salmonella/Citrobacter/Cronobacter/Enterobacter/Morganella
IG0181	Klebsiella pneumonia/Aeromonas
IG0195	Klebsiella oxytoca/Pantoea agglomerans
IG0204	Serratia marcescens
IG0133	Shigella and E.coli
IG0008	Haemophilus
IG0039	Pseudomonas/Morganella morganii
IG0012	Firmicutes (separates on Streptococcus and some Clostridiales)
IG0023	Firmicutes (separates on Listeria, Veillonella and some Clostridiales)
IG0044	Clostridiales and Veillonellaceae
IG0058	Clostridiales and Veillonella
IG0103	Clostridium sporogenes
IG0163	Clostridium butyricum
IG0095	Anaerococcus prevotii
IG0107	Finnegoldia magna
IG0051	Veillonella
IG0042	Gemella
IG0079	Streptococcus (β -haemolytic group A and B, α -haemolytic)
IG0022	Streptococcus (β -haemolytic group B, α -haemolytic)
IG0197	Streptococcus (α -haemolytic)
IG0081	Streptococcus agalactiae and Eubacterium rectale
IG0020	Streptococcus sanguinis and thermophilus
IG0171	Enterococcus faecalis
IG0024	Staphylococcus and Gemella
IG0063	Staphylococcus epidermidis, Eubacterium rectale, Clostridium SS2, Streptococcus agalactiae
IG0053	Lactobacilli
IG0021	Listeria sp. (and E. coli/Shigella)

The present study depended on correlation between age and colonization of Microbiota. (**Fig, 11 and Table, 1**) showed that some probe gave high signals with high values and some gave low signals with low value depend on the got ages, different types of signals in probe for different taxonomic groups of bacteria.

At the age 3 to 120 days Universal bacteria have increased whereas some bacteria have decreased.

The 16S rRNA-gene PCR, that was performed on the DNA extract, gave amplification of the 16S rRNA gene. In the Cover 16S rRNA-gene PCR products. All group get amount of DNA except group7 (**Fig, 2**).

16S rRNA gene microarray approaches to describe the gut microbiota. A major challenge with traditional 16S rRNA gene microarrays is probe specificity, and cross-reactivity between closely related species (Cox *et al.*, 2010). For microarrays this challenge has recently been addressed by tiling probes covering the variable region of the 16S rRNA gene (Rajilic-Stojanovic *et al.*, 2009) and by the use of highly specific single nucleotide primer extension (SNUPE) probes (Eggesbo *et al.*, 2011).

The principle by tiling is that a large number of overlapping probes cover the region of interest, with the combined probe signals providing a relatively good signal to noise ratio. The high specificity of the SNUPE assay is obtained by DNA polymerase based incorporation of a fluorescently labeled dideoxynucleotide (Syvanen *et al.*, 1990). The SNUPE probes are constructed so that the probes hybridize adjacent to discriminative gene positions.

If the target bacterium is present then a labeled dideoxynucleotide is incorporated by the polymerase (Vebo *et al.*, 2011).

The different sample parallels were inconsistently amplified in the Cover All PCR reaction and also the amount of DNA amplified did not reflect back on the originally amount from the DNA

extraction. DNA extraction methods and PCR primers used will always give biases regarding which bacteria that will be identified (Hong *et al.*, 2009).

This PCR was used to amplified 1200 bp region of the 16SrRNA. The size of Cover All PCR product was checked by agarose gel electrophoresis. The band we observed was 1200 bp. (**Fig, 2**) as expected. Therefore quantification of the PCR product was done using PicoGreen.

In the result of PCR product DNA quantification by PicoGreen where each group got same result in every sample only group 7 did not get the same data from there sample because of some mistake in the technique.

Similarly pyrosequencing also addresses for the analysis of gut microbiota. The process utilizes the sequencing primer which hybridized to a single – stranded PCR amplicon that serves as template. The first dNTP is incorporated to the DNA strand by polymerase if it is complementary to the base into the DNA strand. Each incorporation is accompanied by release of pyrophosphate. ATP sulphuryase converts pyrophosphate to ATP in presence of adenosine 5 phosphosulfate (Syvanen *et al.*, 1990). This ATP helps to convert luciferin to oxyluciferin that generates visible light. The light produced is detected by charge coupled device chip and seen as peak in the program. The height of each peak is proportional to the number of nucleotides incorporated. Apyrase, a nucleotide- degrading enzyme, continuously degrades unincorporated nucleotides and ATP (Gharizadeh *et al.*, 2007).

When degradation is complete, another nucleotide is added. The complementary DNA strand is built up as the process continues and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace (Ronaghi, 2001).

The gut microbiota was identified and determinate by the GA-map infant array and 454 pyrosequencing in which significance difference of bacteria. Several studies that reported Bifidobacteria as the months (**Fig, 7**) dominant microbiota of breast-fed infants by several weeks of age (Benno *et al.*, 1984; Favier *et al.*, 2002; Penders *et al.*, 2006 and Stark and Lee, 1982). Result of current research showed that qPCR and pyrosequencing Bifidobacteria are increased in the 10 days to 4 after that the range decreased, that is mean the two methods are all dependable in the main stream .

When comparing two data seta from microarray and qPCR its clear that in group 2411 *B.longum* and *B.not longum* come to the highest value in day 3 while *B.longum* become less in the later

stage (**Fig 4, 5**), while the other type still increase. In group 2206 recorded that some types of *Bifidobacterium* become highest in 4 months (**Fig 9, 10**) and *B.nolongum* without *B.longum* become less after day 10 which means that *B.longum* remain dominant strain in the gut after that time.

There are no difference in the species of *B.longum* and *B.non longum* between IgE sensitive and non-sensitive children (Vebo *et al.*, 2011). This bacterium has a function in the temporal development of the infant intestine and a future aim of research could be to find out more around the connection of *Bifidobacteria* and breast-fed infants in stool samples.

4. CONCLUSION

- 1- The gut dominating *Bifidobacteria* can give more information about the development of microbiota in human gut in further research.
- 2- Understanding the gut microflora may be a milestone for developing strategies for intervening microflora in the reduction of allergic disorder in the developed world.

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