# Tracking population dynamics of *E. coli* strains in a healthy human infant over the first year of life

Sigmund Ramberg

*60 study points*


Thesis for the Master's degree in Molecular Bioscience

UNIVERSITY OF OSLO

05/2016

2016

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Sigmund Ramberg

http://www.duo.uio.no/

Trykk: Reprosentralen, Universitetet i Oslo

# Abstract

Understanding the normal development of the human gut microbiome is of great interest. This is mainly due to possibilities for predicting and preventing disease and developing probiotic treatments. *Escherichia coli (E. coli)* is one of the first organisms to colonize the infant gut, and is used as an indicator organism for changes in the population structure microbiome as a whole. In order to more accurately map the development of the infant gut microbiome, and to prepare for large scale studies in the future, a novel methodology was tested where fragments of the *E. coli* house-keeping genes *malate dehydrogenase* (*mdh*) and *tryptophan synthase alpha subunit* (*trpa*) were amplified from fecal samples taken over the course of the first year of life of a healthy human infant, and sequenced using Pacific Biosciences Single molecule real time (SMRT) sequencing with sample multiplexing. Strains were phylogenetically categorized using database sequences for known reference strains. In this study, eleven distinct *mdh* alleles and eight distinct *trpA* alleles were observed in the infant during the sampling period. In theory, this indicates that at least eleven unique *E. coli* strains were observed to be colonizing the infant over the study period. This is many more than previous studies have observed and is possibly due to the large number of samples from a single infant that were analyzed. All alleles have been previously recorded in the MLST databases for both the *mdh* and *trpA* alleles. However, it was only possible to match four of the *mdh* and *trpA* alleles with each other, using common occurrence in the sequencing data, and thus postulate that they occur on the same genome and represent a unique strain. Of the strains that were identified, we observed populations dynamics with some strains having a dominant position in the *E. coli* population during distinct time periods, separated by transitional periods with higher strain diversity. Some of these shifts in strain composition correlated with environmental factors, such as travel or changes in diet. The procedure successfully allowed for the mapping of the development of the infant gut microbiome with a much higher resolution than previous studies, and allowed for the temporal pinpointing of when changes in *E. coli* strain composition occurs and how strain composition fluctuates in transitional periods. The procedure can easily be adapted to map and compare the development of the early gut microbiome of multiple infants, although further optimization of the procedure would be desirable to improve the signal to noise ratio.

# Acknowledgements

The work reported in this thesis was performed at the Department of Molecular Biosciences, Centre for Ecological and Evolutionary Synthesis, Faculty of Mathematics and Natural Sciences, University of Oslo, with the support of Nils Chr. Stenseth, between fall 2014 and spring 2016.

I would like to thank my supervisors, Pål Trosvik and Eric de Muinck, for their guidance, support, motivation and good humor during my time working with them. I would like to thank Karin Lagesen for being an excellent teacher when I first started to learn programming, for being available for consultation during my research, and for motivating me to pick this project in the first place. I would like to thank Monster Energy Drinks and the Stoic philosopher Epictetus, for helping me keep working when things seemed the most dire. I would like to thank my parents and siblings for always believing in me. Lastly, I would like to thank my wonderful girlfriends, Kristin and Emma, for the endless support and love they have shown me these last two years, and for knowing when to leave me alone so I could actually get some work done. You two are my life.

Sigmund Ramberg,
Oslo, May 2016

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# 1 Introduction

## 1.1 Human microbiome

### 1.1.1 Early colonization

In human infants, the gut is commonly thought to be sterile as long as the fetus is suspended in the amniotic fluid, and initially colonized by microorganisms derived from initial exposure to the mother's microbiome during the process of birth, and then later affected by diet and other environmental factors that alter the composition of species and strains present (Gritz et al. 2015).

The composition of the neonatal gut microbiome and how this changes as a result of environmental triggers is of great potential interest from a health perspective, both since microbiological challenges to the developing immune system are thought to be important in resistance to later disease (Langhendries et al. 1998), and because probiotic organisms can help maintain a healthy metabolism during a critical developmental phase (Parracho et al. 2007).

Colonization of new bacteria in the gut microbiome is influenced by the pre-existing composition of species, since established species or strains might take up critical nutrients or create favourable or unfavourable conditions for other organisms. Developing gut microbiomes in young infants are also highly responsive to environmental factors. Birth by caesarean section (Neu et al. 2011), hygiene conditions during the birth, early diet, and antibiotics use by the mother or infant may all have significant effects on the development of the microbiome, and in turn the development of the immune system and general health of the infant (Gritz and Bhandari 2015).

### *1.1.2 E. coli*

*Escherichia coli* is a gram-negative bacteria that occupies the niche of the most common facultative aerobic organism in the gut of vertebrates (Berg 1996), and has become one of our best characterized model organisms, being used extensively as a gene expression system. Although recombination between different strains occurs at quite a high rate in nature, such recombination occurs mostly at specific hotspots, and major genome rearrangements are rarely, if ever, observed (Milkman et al. 1990, Touchon et al. 2009). While this allows for species-wide adaptations in certain traits to occur, it also means that for the majority of their genome, *E. coli* has a clonal population structure, with different strains possessing groups of different genes allowing them to adapt to their specific niche (preferred host organism or life-stage, for example) (Herzer et al. 1990, Gordon et al. 2003).

When inside a host organism it most commonly adopts a commensal lifestyle, collecting nutrients from the mucus layer covering the epithelial cells throughout the digestive tract (Freter et al. 1983). However, some strains also have probiotic or pathogenic effects, or are known to adopt such under certain conditions. These have been suggested to be in large part coincidental; their aerobic metabolism lowers oxygen content in the gut and creates favourable conditions for other desirable microorganisms, and they generate toxins to remove bacteriophages and other organisms that may also be harmful to the host. However, such defences, or other proteins that allow for more efficient colonization of the gut of a specific host organism may lead to pathogenic effects when introduced to another organism (Tenaillon et al. 2010).

In humans, *E. coli* is present in larger amounts per gram of faeces than in most other studied domestic and wild animals, and it is one of the first bacterial species to colonize the intestine during infancy, being transferred to the infant from the mother and maternity nursing staff (Bettelheim et al. 1976, Penders et al. 2006). Because of this, a reduction in early colonization by *E. coli* is observed in industrialized countries, which has been attributed to more stringent hygiene practices in hospitals and the general population and to the increase of c-section births which has been shown to reduce *E. coli* transmission from mother to infant (Nowrouzian et al. 2003).

The *E. coli* population in an individual tends to have one dominant strain which persists over a period of time, although over longer timespans the dominant strain changes in response to environmental factors, such as changes in diet, antibiotic use, exposure to new strains, or potentially other unidentified factors leading to a change in the microbiome as a whole (Caugant et al. 1981).

After the first two years of infancy, *E. coli* concentration in the human gut reaches $10^8$ colony forming units (cfu) per gram of faeces, where it remains stable into adulthood and for the majority of the host's lifespan (Mitsuoka et al. 1973). Adult humans are generally resistant to induced colonization of new *E. coli* strains, while infants are more susceptible (Poisson et al. 1986). Experiments in mice have shown that certain strains of *E. coli* will not colonize the intestines of mice with pre-existing gut floras, but will colonize the intestines of mice treated with streptomycin, and, having then established itself in the mouse gastrointestinal microbiome, will persist after the reintroduction of normal gut flora (Freter, Brickner et al. 1983), suggesting that resistance to colonization in adults can be at least in part attributed to established strains out-competing foreign strains being introduced to the microbiome.

## 1.2 Mapping bacterial population dynamics

### 1.2.1 Bacterial typing techniques

In any study where the aim is to study bacterial population dynamics, or the properties of a specific strain under particular conditions, it is essential to have a reliable method of identifying which types of bacteria are present in a sample. In addition being classified into species, microorganisms are typically also classified into strains, which are populations of organisms genotypically distinct from isolates of other strains, with specific phenotypes, but which are not different enough to be classified as different species.

Traditionally, since Robert Koch discovered how to make pure cultures in the 19th century, genus, species, and sometimes even strains have been identified through making cultures of bacterial colonies from samples, and then studying the phenotypic properties of these cultures, such as antibiotic resistance, serotype, phage type, staining characteristics, metabolism and nutritional requirements, and morphology of colonies and cells. The type of bacteria is then determined by comparing these traits against isolate databases, or using specialized kits that automatically interpret your results to determine probable species or strains (Foxman et al. 2005).

These methods of bacterial typing have some limitations that made them difficult to use for studies involving large numbers of samples or requiring a high degree of discriminatory power. They all rely on being able to generate growth cultures, which can be time consuming, depending on the growth rates of the organism, and introduces bias already in the first step of analysis, since some types of bacteria are easier to culture in vitro than others, meaning results may not accurately represent the composition of the sample. In addition, phenotypic analysis does not allow you to distinguish genotypically separate strains that share the phenotypes you are looking at, nor provide a solid basis for building phylogenies of closely related species and strains, which can be problematic if observed phenotypes do not match exactly with any characterized strains. Lastly, the methods with the highest discriminatory power are limited in how broadly they can be applied. For example, phage typing is reliant on having access to strain specific bacteriophages for all the strains in your sample, if you wish to map it out completely (Foxman, Zhang et al. 2005).

Due to sequencing and other molecular biology techniques that were developed in the 1970s and 1980s, it is now becoming increasingly common and viable to use techniques that do not rely on studying the phenotypes of cultured bacteria, and instead establishing the genotype through enriching and studying all or parts of the genetic material isolated from cultures or directly from environmental samples (Foxman, Zhang et al. 2005). Examples of some of these techniques are:

Pulsed Field Gel Electrophoresis, first developed by David C. Schwartz and Cantor in 1984, is a method for performing genetic fingerprinting using DNA digested with restriction enzymes generating large fragments, and running the samples through a gel with three

alternating axes of applied current, allowing for efficient separation of larger fragments than is normally possible with gel electrophoresis. The resulting fragments generated by specific enzymes or combinations of enzymes are distinct for different genera, species, and often strains if they display polymorphisms at the sites targeted by the restriction enzymes. Some strains are not typed easily by this method due to DNA degradation during electrophoresis, and it does not provide sufficient sequence information for meaningful phylogenetic analysis (Schwartz et al. 1984, Johnson et al. 2007).

Ribotyping is another typing method based on isolating restriction fragments containing the 16S and 23S rRNA sequences, which are conserved in all bacterial species, but with species specific variations. The types of fragments present in the samples are then visualized using fluorescent probes. The process is quite quick, can be automated, and many species have been characterized, but the equipment is relatively expensive (Grimont et al. 1986).

DNA Microarrays is a typing technique that relies on using what is commonly known as a biochip: A surface to which a collection of DNA probes have been attached in an ordered pattern, which produce a light signal when they bind to a complementary sequence. While this method is often used to study gene expression using isolated mRNAs, it can also be used to type bacterial strains using chips that have been prepared with variants of specific marker genes, thus allowing specific strains or species to be identified, depending on the genes and variants selected. Typing chips exist for a number of bacterial pathogens, but availability, cost, and time needed for post-analysis can be limiting factors in applicability (Bumgarner 2013).

Although the above mentioned techniques provide some genetic information, they rely on identification of specific pre-selected genetic markers, and do not provide as detailed information as sequencing based techniques, which allow for more accurate studies of strain phylogeny (Johnson, Arduino et al. 2007).

Multilocus Sequence typing (MLST) is a genotyping method relying on amplification and sequencing of small fragments (typically 400-500bp) of specific highly conserved genes with small variations between strains, using schemes of genes and primers often defined by the isolate databases specific to the species you are studying. Since typing schemes are species specific, it does not allow you to map the entire genetic content of the sample, but the method has high discriminatory power between different strains of specific species, with cost, time and discriminatory power all increasing with the number of genes interrogated. MLST databases exist for a large number of human and plant pathogens (Maiden et al. 1998, Johnson, Arduino et al. 2007).

Ideally, one would perform Whole Genome Sequencing of the genetic material in samples or isolates, allowing us to completely unambiguously identify all strains present, and reducing the need to grow pure isolates to avoid conflating results from multiple different strains. Although this is becoming increasingly viable as sequencing technology becomes more efficient and affordable, it is still considered too expensive and time consuming for most

studies, and the vast amounts of output data requires bioinformatics techniques, databases, and computing power that are not readily available. Therefore, many researchers decide to use other techniques that best balance timescales, budgets, and discriminatory needs (Dark 2013).

## 1.3 Polymerase Chain Reaction

In genetics and molecular biology, it is often useful or essential for a researcher to be able to amplify the specific DNA sequences in a sample. This is important for many different applications such as assaying samples for the presence of a target DNA sequence, visualizing target sequences with gel electrophoresis, preparing DNA for sequencing, amplifying sequences for insertion into cloning vectors, and many other applications. Polymerase Chain Reaction (PCR) is a common molecular biology technique in which a defined piece of DNA is amplified in vitro using DNA polymerase. A method for amplifying short DNA fragments was described as early as 1971 in a paper by Kjell Kleppe et al. (Kleppe et al. 1971), but credit for the modern PCR protocol is usually given to Kary Mullis, who patented it in 1986 (Google 1986) and received the Nobel Peace Prize in chemistry for it in 1993 (Abdulkareem 2014).

The process relies on repeatedly changing the temperature of the reaction, and as such a heat-stable polymerase, such as the Taq-polymerase from Thermus aquaticus, is used in nearly all instances. The process begins with heating the sample with the polymerase and other reagents in order to denature the double-stranded DNA in the sample. The temperature is then lowered to allow for the annealing of primers to the single-stranded DNA. Primers are small DNA fragments that are complimentary to a section that one wishes to amplify on the template, typically one for the sense strand and one for the anti-sense strand. If the temperature is lowered too much during this step, the primers may bind to sections that are not perfect complements, causing the amplification of regions other than the intended target (Saiki et al. 1988).

Once the primer has hybridized to the template strand, the temperature is raised to a level close to the optimum working temperature for the polymerase used in the reaction. The polymerase then binds to the primer-template complex and extends the primer in its -3' direction using deoxynucleoside triphosphates which were added to the reaction mix, until it reaches the end of the template. Then the temperature is raised further to denature the generated double-stranded DNA molecules, and the cycle repeats, with the new strands, containing the sequence from one of the primers to the end of the template molecule, acting as templates for the next round of copying, in addition to the original templates. Since the amount of original DNA in the sample remains constant throughout the reaction, but the fraction of DNA where one or both ends terminate in the region matching the primers, the likelihood of primers binding to a template ending at the desired points increases with each cycle, until the vast majority of DNA in the reaction contains only the desired region of DNA. The reaction continues until manually terminated, or until all primers or nucleotides have been used up, or all the enzyme has been denatured, at which point no further amplification is possible (New England Biolabs).

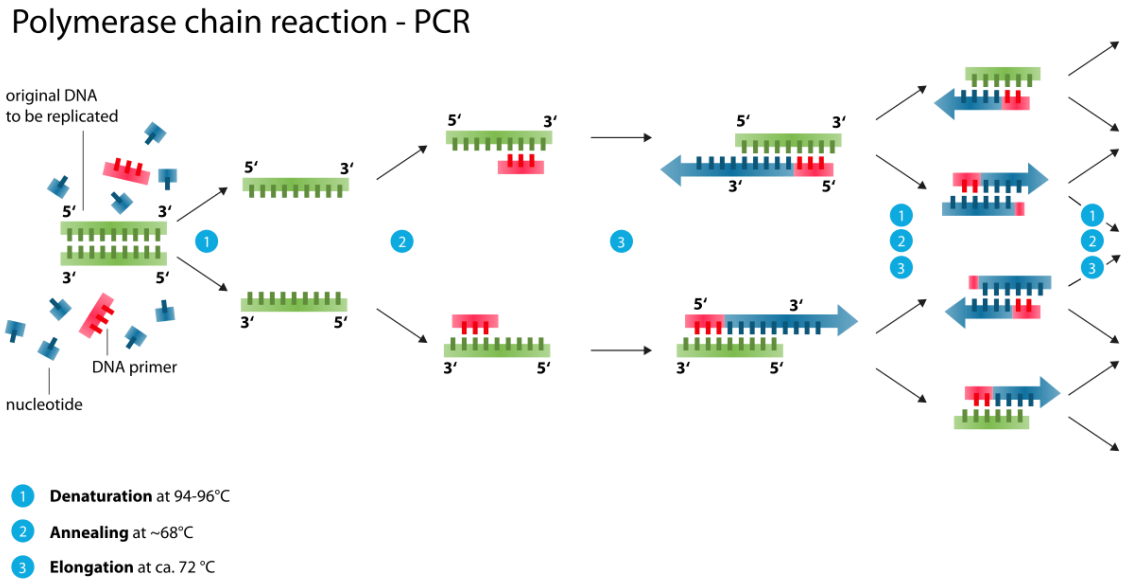


Figure 1. Schematic drawing of the PCR-cycle, by wikipedia user Enzoklop, used under the Creative Commons Attribution-Share Alike 3.0 licence.

After running a PCR reaction, it is common to check if the expected fragment has been generated by separating the contents of the sample by weight and length using horizontal submerged gel electrophoresis. DNA migrates through an agarose gel submerged in buffer, using an electric current to attract the negatively charged DNA to the anode at speeds that vary with the length of the fragment, with smaller DNA fragments migrating faster than larger DNA fragments. During migration the DNA binds to an intercalating agent that binds double stranded DNA, allowing visualization of DNA bands upon irradiation with e.g. UV light. The gels are also loaded with a DNA ladder; a collection of fragments with known lengths, which can be used to estimate the length and weight of fragments in the sample by comparison with the ladder (Lee et al. 2012).

Multiple factors can be optimized to improve PCR yields for samples that are difficult to amplify. Temperatures can be optimized to decrease the rate of non-specific binding of primers. The buffer for the reaction may be changed to facilitate amplification of GC-rich sequences. If the reaction is occurring, but at a lower rate than expected, yields may be increased simply by increasing the number of cycles in the PCR program, although this may introduce amplification bias. If the primers are binding to each other rather than the template due to accidental complementarity, this will result in the creation of small fragments called primer-dimers, which show up in the gel. To avoid this, different binding regions can be selected when designing primers, in order to reduce complementarity. Dimethyl Sulfoxide can be added to the reaction to decrease the formation of secondary structures in the DNA that inhibit the binding and elongation of primers, such as hairpin loops (Chakrabarti et al. 2001). Lastly, if the sample is suspected to contain impurities that interfere with polymerase activity, and further purification is not an option due to limited sample volume, Bovine Serum Albumin (BSA) may be used to increase the stability of the polymerase and prevents it from adhering to the reaction tubes or pipette tips (Farell et al. 2012). Additionally, Mg2+ ions act as essential catalysts during PCR, but too high concentrations can increase the rate of non-specific primers and decrease the fidelity of the reaction (New England Biolabs).

### 1.3.1 Primer barcoding and sample multiplexing

It is often desirable to pool and analyze multiple samples in one sequencing run. In that case the expected read number should be high enough to provide sufficient information about each sample. This is referred to as multiplex sequencing. However, since there is no way to tell which sample a sequence comes from in the sequencing output if they are all in the same reaction, the sequences themselves have to be altered in some way to contain this information. This is done by adding what is called an index sequence to the end of one or both primers used when preparing the sample.

An index sequence is an arbitrary sequence that has been assigned to indicate one or more specific source samples. It should ideally be short, to avoid interfering with the PCR reaction, non-complimentary to the template to avoid PCR bias, and be sufficiently different from other index sequences used to avoid misidentification as another sample as a result of read errors. If both primers contain an index sequence, it becomes possible to reuse individual primers on a different sample by pairing it with a different index sequence on the opposite end of the fragment, and representing each sample by the combination of index sequences. The number of possible samples covered by a primer set then increases by the square of the number of primer pairs, rather than being equal to the number of indexed primers (Parameswaran et al. 2007, Pacific Biosciences 2015, Maki et al. 2016).

## 1.4 DNA Sequencing

DNA sequencing is the process of determining the order of nucleotide bases in a piece of DNA, and it has numerous applications in biological research, medicine, and forensics. Sequencing is being used to map and study the genomes of organisms; in studies of protein expression and function; identifying organisms in environmental samples; finding phylogenetic relationships between organisms; diagnosing hereditary diseases and potentially judging the effectiveness of different treatments in what is known as personalized medicine; and determining paternity or performing forensic identification, to name a few uses.

The first methods for DNA sequencing were developed in the 1970s. One of these was Maxam-Gilbert sequencing, also known as chemical sequencing, developed by Allan Maxam and Walter Gilbert in 1977. Maxam-Gilbert sequencing works by treating different sets of identical, 5-end radioactively labelled DNA fragments with chemicals that selectively cause breaks at specific nucleotides (G, A+G, C, and C+T). The resulting fragments from the four reactions were put through size-separating gel electrophoresis, and visualized with film sensitive to the radiation from the labels, thus making it possible to determine the DNA sequence (Pareek et al. 2011).

The very first method for DNA sequencing was developed by Ray Wu in 1970, which relied on DNA polymerase mediated primer extension and labelling of nucleotides. This formed the basis for the most successful of the 1st generation sequencing methods, Sanger sequencing, or the chain-termination method, which was developed by Frederick Sanger in 1977. The process works by synthesizing a new DNA strand using the DNA to be sequenced as a template, and including low concentrations of modified nucleotides in the reaction mix that terminate the elongation process. Originally, the sequence was determined using four separate reactions, similar to Maxam-Gilbert sequencing, and each reaction contained only the modified variant of one of the four bases. Later, terminating nucleotides with fluorescent dyes were developed, making it possible to determine the identity of a nucleotide just by looking at the resulting bands after size-separation, and negating the need for separating the process into four different reactions. Due to relying less on radioactive labelling and toxic chemicals, and because of its relative ease of use, Sanger sequencing became the most commonly used method of sequencing in the 80s and 90s and was used in the first-generation automated sequencing machines. Although it has today in large part been replaced by other methods, it is still used in smaller scale projects and to verify results from newer sequencing methods (Pareek, Smoczynski et al. 2011).

Starting in the 90s, several methods were developed that allowed for the sequencing of large numbers of DNA molecules in a single reaction, and at a much lower cost per base than Sanger sequencing. These methods are collectively referred to as Next Generation Sequencing methods, and some examples include:

SOLiD sequencing, developed by Applied Biosystems in 2008, which works by ligation of amplified DNA fragments to prepared oligonucleotide probes attached to a glass surface, as opposed to sequencing by synthesis, as in Sanger sequencing. The probes include all possible variations of oligos of a certain length, and since the fragments to be sequenced preferentially ligate to probes with complementary sequences, mapping which probes are ligated to allows for the determination of the fragment sequence. While the method has a high accuracy and a relatively low cost per base, resulting reads are very short, between 50 and 100

base pairs, and it is very time consuming, with a single run taking up to two weeks (Mardis 2008, Pareek, Smoczynski et al. 2011, Liu et al. 2012).

Ion Torrent Sequencing, developed and released by Ion Torrent Systems Inc. in 2010, is a synthesis based sequencing technology that works by detecting hydrogen ions released during the process of synthesis. This is achieved by attaching the DNA to be sequenced inside a tiny well in a semiconductor surface, and flooding the well with a single type of nucleotide in turn. If polymerisation occurs, hydrogen ions are released which generates a detectable electrical signal. If multiple identical nucleotides are attached in a row, the signal strengthens, though large homogenous regions can make it difficult to get an accurate read on the exact number of nucleotides added in a single reaction step. The method allows for sequencing of DNA fragments up to 400 base pairs in two hours, and the machine is less costly than other alternatives, though the cost per base is higher than most other Next Gen sequencing methods (Mardis 2008, Pareek, Smoczynski et al. 2011, Liu, Li et al. 2012, Quail et al. 2012).

Illumina Dye Sequencing is a sequencing technology originally developed by Solexa Inc. in the late nineties. DNA to be sequenced is fragmented using transposomes, and adapters are added to each end of the fragments. These adapters and then modified to allow the fragments to bind to specially prepared chips containing anchored oligonucleotides, and then amplify them in such a way that thousands of copies of the fragment are generated in spatially isolated sections of the chip, generating what is referred to as DNA clusters to amplify the signal during the sequencing step. Complimentary strands to the fragments are then sequenced using modified nucleotides, that limit the sequencing process to one base at a time, and which cause clusters to generate diffferent light signals with each nucleotide added. Time to run and number of reads varies greatly depending on the model used, with the HiSeq X providing up to 3 billion reads. Equipment for Illumina sequencing is generally quite expensive, and the reaction requires higher concentrations of input DNA than other Next Gen methods (Mardis 2008, Pareek, Smoczynski et al. 2011, Liu, Li et al. 2012, Quail, Smith et al. 2012).

454 Pyrosequencing, developed and released by 454 Life Sciences in 2005, is another sequencing by synthesis based method where the output signal is generated using luciferase, which is activated during sequence elongation. In order to prepare for sequencing, template DNA is amplified in a process called emulsion PCR, where the DNA is amplified inside water droplets suspended in oil, with each droplet containing only a single kind of sequence, and the resulting beads being deposited in separate microreactors. Since the procedure does not rely on modified nucleotides to prevent multiple bases being added at once, homopolymeric regions of DNA are distinguished only by the strength of the output signal, and it can be difficult to tell apart longer stretches of DNA containing only one type of nucleotide. The method also has a high run cost per sequenced base, but can produce reads up to 700 bp in length in 24 hours, with very high accuracy (Mardis 2008, Pareek, Smoczynski et al. 2011, Liu, Li et al. 2012).

Single Molecule Real Time sequencing is another synthesis based method developed by Pacific Biosciences and released in 2011. The method is based on DNA polymerases attached to the bottom of small chambers called Zero-mode waveguides, which allow for the activation of fluorescent dyes within a very small volume at the bottom of the chamber, and nucleotides with fluorescent dyes attached in such a way that they are cleaved off by the DNA polymerase during integration in the growing strand. While being integrated, the individual dyed nucleotides are kept in place by the polymerase at the bottom of the chambers much

longer than when free-flowing, and this generates a light signal detectable by the sequencing machine. An individual SMRT chip contains a large number of these ZMW chambers, which allows for a large number of parallel reads. Reads per run tends to be lower than many other methods however, which results in a moderate throughput compared to other fast methods with millions or billions of reads per run. Although the method has a higher error rate for individual reads than other methods, this can be compensated for using a technique called circular consensus sequencing, where hairpin adaptors are ligated to the ends of the template to be sequenced, creating a circular piece of DNA which is read multiple times by the same DNA polymerase (Travers et al. 2010). Results can then be filtered by read quality, and the method allows for much longer reads than other methods, usually between 10000 and 15000 base pairs, with a relatively low runtime and cost per base. Since the method depends on semi-direct observation of the polymerase during nucleotide integration, variations in integration speed can be used to determine the methylation state of specific nucleotides (Mardis 2008, Pareek, Smoczynski et al. 2011, Liu, Li et al. 2012, Quail, Smith et al. 2012).

Figure 2. Schematic representation of SMRTBell template used for PacBio Circular Consensus sequencing.

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## 1.5 Aim of study

The goals of the project were:

1. Design and test out bar-coded primers for *E. coli* housekeeping genes from two different MLST schemes.

2. Develop a higher throughput methodology to allow for the typing of hundreds of *E. coli* samples.

3. Amplify and sequence the selected *E. coli* housekeeping genes from DNA isolated from fecal samples from a human infant, taken at frequent intervals between ages 0 and 12 months.

4. Identify, categorize, and quantify *E. coli* strain types in the samples using the sequencing data, and determine how the strain composition and relative abundance of the gut changes over time, as well as identifying potential environmental factors or phenotypic properties that might contribute to such changes of the composition of the microbiome.

This project is related to previous work done by Eric de Muinck, where he compared the strain composition of *E. coli* in the gut microbiome of a group of human infants over five time points (2d, 4d, 10day, 4months, and two years)(de Muinck et al. 2011). The methodology developed here allows for MLST typing in a multiplexed format of at least one hundred samples per PacBio sequencing run. In this thesis we applied this methodology to follow fine scale *E. coli* changes over time in a single infant over the first year of life. This can be considered a proof of concept for future research in which strain dynamics of many different species of host bacteria can be followed in populations or in individuals at fine time scales.

# 2 Experimental

## 2.1 Materials and reagents

All PCR reactions were performed using Phusion DNA Polymerase and Phusion HF or GC Buffer from the Thermo Fisher Scientific Phusion High-Fidelity DNA Polymerase kit. 2 mM dNTP, MiliQ H2O, and 10 mg/ml BSA.

PCR results were visualized by electrophoresis on 1% Agarose gels with Gel Red fluorescent DNA stain, run with 1x TAE buffer. Samples were loaded using Thermo Fisher Scientific 6X Massruler loading dye, and results compared against Low Range Thermo Fisher Scientific FastRuler DNA Ladder.

DNA concentrations were measured with a NanoDrop spectrophotometer. Before final pooling of samples, DNA concentration was measured with a Qubit 2.0 fluorometer using reagents from the Thermo Fisher Scientific Qubit dsDNA BR (Broad Range) assay kit.

Before submission for sequencing, pooled samples were purified using the Qiagen QIAquick PCR Purification kit, together with 96% ethanol and 3M sodium acetate.

### 2.1.1 Samples and standards

For the PCR reactions, DNA isolates from strains in the ECOR collection were used as template for the positive controls. The strains used were: ECOR 19, 31, 34, 40, 42, 43, 60, 66, and 69. In addition fecal DNA from a healthy adult isolated using the Qiagen Stool Kit was used as controls to test if the extraction protocol caused samples to contain contaminants that might influence PCR.

After initial testing, 16S primers 806r and 515f (Caporaso et al. 2012) were used as a control for all samples.

### 2.1.2 DNA isolates

Fecal samples were collected over one year from a healthy newborn infant according to REK agreement (2014/656). Samples were immediately frozen at -20°C pending transfer to a long term storage facility at -80°C. Total DNA from fecal samples was extracted using the MO BIO PowerSoil 96 well DNA isolation kit.

## 2.2 Designing and testing primers

In a previous study, it was found that sequencing of a fragment of a single house-keeping gene, malate dehydrogenase (*mdh*), was in many cases sufficient to determine the phylogenetic group of *E. coli* strains from fecal samples from infants, and did not show large deviation from strain identification performed with a full 7-gene MLST. In order to test if this trend holds true for other MLST schemes, and to produce additional data for potentially ambiguous results, it was decided to sequence an additional fragment. In this case we used the tryptophan synthase alpha subunit (*trpA*) house-keeping gene, which is used in the *E. coli* MLST scheme developed by the Pasteur Institute.

In order to simplify the design process, it was decided to use only the last 20 bases on the three prime ends of the *trpA* primers, so that all primers used for both genes were of roughly equal length, with exception of the *mdh* forward primer, which was three bases longer. *In silico* PCR simulation was used to confirm that shortening the primer sequences did not lead to off-target binding.

Index sequences were generated using a custom script coded in Python 2.7 (appendix 1), which allowed for the generation of sequences of any specified length, and filtering to ensure that each sequence had any desired level of difference from each other sequence in the list. Since errors can occur during sequencing, it was desirable for each index sequence to be as different from every other index sequence as possible in order to reduce the risk of misidentification during demultiplexing. The length parameter in the script was set to generate indices of 5 nucleotides, where each had at least three bases different from every other. This resulted in a list of 64 distinct indices. (appendix 2, table 16)

14 distinct indices of the forward primers were chosen from the table for each gene and 10 of the reverse primers, resulting in 140 distinct combinations of primers for each gene. Additionally, in order to avoid amplification bias in cases where the index sequence happened to match the five prime upstream region of the non-indexed primers, a two-base linker region, designed to not match the upstream sequences of the non-indexed primers, was included between the template binding region of the primers and the index sequences. (Appendix 2, table 17)

The resulting set of 48 primer sequences were submitted to Integrated DNA Technologies for synthesis. Primers were generated in quantities of 25 nmoles through Oligonucleotide synthesis, deprotected, desalted, and dried for shipping.

In order to confirm that the primers had been synthesized correctly, and that the index sequences did not interfere with PCR activity, all 280 primer combinations were tested on *E. coli* control templates before attempting to amplify the fecal sample DNA.

Following the recommendations from the Thermo Fisher Scientific Phusion Polymerase documentation (Thermo Fisher Scientific 2013), original reaction mixes and PCR program used were as follows:

| 1x 50 µl PCR reaction mix | |
|---|---|
| MiliQ $H_2O$ | 27,5µl |
| 5x HF buffer | 10µl |
| 2mM dNTP | 5µl |
| 10µM Forward primer | 2,5µl |
| 10µM Reverse primer | 2,5µl |
| Phusion DNA Polymerase | 0,5µl |
| Template DNA | 2µl |

| 1x 20 µl PCR reaction mix | |
|---|---|
| MiliQ $H_2O$ | 10,8µl |
| 5x HF buffer | 4µl |
| 2mM dNTP | 2µl |
| 10µM Forward primer | 1µl |
| 10µM Reverse primer | 1µl |
| Phusion DNA Polymerase | 0,2µl |
| Template DNA | 1µl |

| PCR program | | |
|---|---|---|
| Denaturation | 98°C | 30 seconds |
| 30 cycles: | 98 °C | 10 seconds |
| | 55 °C | 30 seconds |
| | 72 °C | 30 seconds |
| Final extension | 72 °C | 7 minutes |
| Hold | 10 °C | Indefinitely |

Tables 1-3. Recipes for PCR reaction mixes of different volumes, and PCR program used in initial experiments.

Alterations to the reaction mix and PCR program are noted as they were implemented in the testing regimen. To streamline reaction setup, master mixes were made containing all reagents except for primers and template, multiplied by the number of reactions in the experiment, and distributed into the PCR tubes. Template and primers were added to individual tubes as dictated by the experiment setup. After PCR, 10 µl of PCR product mixed with 2 µl Massruler loading dye (Thermo Fisher Scientific 2012) for each reaction was loaded onto separate wells on a 1% agarose gel, next to 5 µl Fastruler low range DNA ladder (Thermo Fisher Scientific 2012). This was reduced to 5 µl of PCR product with 1 µl Massruler loading dye after the first two experiments, as the excessive amount of DNA loaded caused the bands to form large blobs rather than narrow bands when smaller wells were used to run a higher number of samples per gel.

Elctrophoresis was performed at 100V for 30 minutes, and the resulting bands were visualized using the Syngene GeneGenius BIO imaging system.

In the first experiment, the primer combination *mdh* Forward 1/Reverse 1 was compared to unindexed *mdh* primers as a positive control. For each primer combination, four 50 µl reactions were prepared: For each of the temples, ECOR66 and ECOR69, a reaction with the template and a negative control without the template were prepared. Since the two negative controls were identical, one was removed in future experiments as it was considered redundant.

| Reaction nr. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Primers | *MDH* Control | | | | *MDH* F1-R1 | | | |
| Template | None | ECOR66 | None | ECOR69 | None | ECOR66 | None | ECOR69 |

Table 4. Experimental setup for prototype primer testing scheme.

All negative controls displayed no bands during visualization. Test reactions had strong bands in the 600-700 base pair region as expected, but the indexed primers had bands indicating smaller fragments as well. These were thought to be caused by primer dimerization

or other non-specific hybridization due to suboptimal annealing temperatures, since the ideal temperature had yet to be confirmed experimentally. (appendix 3, figure 16)

Using a similar setup, primer combinations *MDH* F2-R2, F3-R3, F4-R4, and F5-R5 were tested with ECOR66 and ECOR69 as templates, using the unindexed *mdh* primers as a control, and having one negative control for each primer combination. All negative controls showed no bands, positive controls displayed bands of expected size as previously, and the test reactions displayed expected bands and smaller bands as in the previous experiment. (appendix 3, figure 17.)

In order to test all primer permutations in a reasonable time frame, a massive upscaling of the experiment was performed: Each run consisted of a multiple of 16 reactions, comprising forward primers 1-14 with a specific reverse primer, and a negative and positive control with the unindexed primer. For each set of 16 reactions, DNA from a randomly picked ECOR isolate was used as template, as the primers should ideally work regardless of the strain used, and the supply of individual DNA isolates was limited.

First run with the large scale setup covered all combinations for *mdh* reverse 1, reverse 2, and reverse 3. For reverse 1 and 3 sets, all test reactions displayed expected bands, and negative control displayed no bands, and positive control displayed expected band. For the reverse 2 set, multiple test reactions showed no bands, and the negative control had a band in the same range as the positive control. This was attributed to pipetting error, and the set was redone as part of the next run. (appendix 3, figure 18.)

Second run with the large scale setup covered all combinations for *mdh* reverse 2, reverse 4, reverse 5, reverse 6, and reverse 7. All positives displayed expected bands, and all negative controls displayed no bands. All test reactions displayed expected bands except for the following: F11-R4, and F13-R7. (appendix 3, figure 19.)

Third run with the large scale setup covered all combinations for *mdh* reverse 8, and all combinations for *trpA* reverse 1-8. Since no unindexed primers were available for *trpA*, the following primers were used as controls:

- For reverse 1 set, F8-R1,
- For reverse 2 set, F8-R2,
- For reverse 3-6 sets, F2-R2,
- 6 has no negative control,
- For reverse 7, no controls,
- For reverse 8, F5-R8.

The majority of the samples produced the expected bands, with the following exceptions:

*TrpA* F8-R1, F1-R6, F13-R6, and F6-R7 displayed none or weak bands. The latter half of R8 displayed no bands, possibly due to low amounts of loading dye while the samples were loaded onto the gel. Due to a pipetting error, both positive and negative controls for *trpA* reverse 3 and reverse 4 contain template. (appendix 3, figure 20.)

In the next run, the *trpA* reverse 8 set was run again on the agarose gel. In addition, the PCRs were performed again for the following primer combinations that had previously failed: *mdh* F11-R4, *mdh* F13-R7, *trpA* F7-R1, *trpA* F1-R6, *trpA* F13-R6, *trpA* F6-R7. Finally, to check if contaminants in DNA isolated from fecal samples rather than pure cultures would interfere with PCR, randomly picked primers for *mdh* and *trpA* were tested using increasing concentrations (1, 2, 3, and 4 µl) of two fecal DNA samples, P1 and P2, attained from a healthy adult and isolated using the Qiagen Stool Kit. Unindexed primers were used for positive and negative controls for *mdh*, while the *trpA* set only had a negative control.

Of all the redone tests, the only ones not successful were *trpA* F1-R6 and *trpA* F13-R6. It was decided that 110 successful primer combinations was sufficient to advance testing, and to leave the testing of the reverse 9 and 10 primers for later should the need arise. From the fecal DNA tests, P1 gave positive results across the board, though much weaker than from the ECOR DNA, while P2 produced no bands in all cases. (appendix 3, figure 21.)

When beginning tests with actual sample material, it was decided to use 20 µl reactions, due to limited availability of template. Due to decreased band strength with fecal DNA, it was decided to increase the number of PCR cycles to 35, and to replace 0,8 µl of H2O in the reaction mix with bovine serum albumin.

A set of randomly picked samples were tested against a set of randomly picked *mdh* and *trpA* primers from the set of those confirmed to work with ECOR DNA. Unindexed *mdh* primers were used as positive and negative controls, using one of the samples (Day 281) as template. Positive control had one band of expected size, negative control had no bands. (appendix 3, figure 22.)

| Sample | *mdh* primers | *mdh* results | *TrpA* primers | *TrpA* results |
|---|---|---|---|---|
| Day 226 | F14R8 | Smear | F6R2 | Band |
| Day 214 | F7R4 | Faint bands | F9R2 | Faint band |
| Day 225 | F9R1 | Band | F7R3 | Band |
| Day 246 | F13R6 | Blank | F1R8 | Band |
| Day 350 | F10R7 | Blank | F9R4 | Blank |
| Day 359 | F10R6 | Faint bands | - | - |
| Day 361 | - | - | F2R6 | Blank |
| Day 281 | F3R4 | Band | F6R6 | Blank |

Table 5. Experimental setup for test with randomly picked samples and primers.

In order to further increase amplification reliability, gradient PCR with annealing temperatures between 50°C and 60°C was performed using ECOR34 DNA diluted hundredfold with *mdh* primers F1R1, and P1 fecal DNA *trpA* primers F1R1, in hope that lower template concentrations would make the bands weak enough to pick an optimal upper temperature. Despite this, the resulting bands were strong across the board, and did not show significant decrease with higher annealing temperatures, as would be expected. However, off-target products like primer dimerization decreased with increasing temperatures, and it was decided to increase the annealing temperature to 58°C in future runs. (appendix 3, figure 23.)

In order to estimate the lower detection limit of the primers, a ten-fold dilution series of ECOR34 DNA, starting at 1 and ending at 1/10000000, was used as templates for *mdh* F1R1, *trpA* F1R1, and 16S primers 515F and 806r. For *mdh*, band strength dropped

significantly at 1/1000000 dilution, while in *trpA* and 16S a similar drop occurred at 1/100000 dilution. Using Nanodrop, starting concentration for DNA in the ECOR34 solution was measured to be ~28ng/µl. (appendix 3, figure 24.)

Based on this, the lower detection limit for the *mdh* primers is estimated to be in the region of 0,028µg/µl, while the lower detection limit for the *trpA* and 16S primers is estimated to be in the region of 0,28µg/µl

## 2.3 Sample amplification

Following the results of the testing of the indexed primers, the following scheme was used to amplify MLST targets from the fecal DNA samples.

All PCRs were performed using the reaction mixture and PCR program described in table 6.

| 1x 20 µl PCR reaction mix | |
|---|---|
| MiliQ $H_2O$ | 10µl |
| 5x HF buffer | 4µl |
| 2mM dNTP | 2µl |
| 10µM Forward primer | 1µl |
| 10µM Reverse primer | 1µl |
| 10mg/ml BSA | 0,8µl |
| Phusion DNA Polymerase | 0,2µl |
| Template DNA | 1µl |

| PCR-program | | |
|---|---|---|
| Denaturation | 98°C | 30 seconds |
| 35 cycles: | 98 °C | 10 seconds |
| | 58 °C | 30 seconds |
| | 72 °C | 30 seconds |
| Final extension | 72 °C | 7 minutes |
| Hold | 10 °C | Indefinitely |

Table 6. Recipe for PCR reaction, and PCR program used during sample amplification.

To streamline reaction setup, master mixes were made containing all reagents except for primers and template, multiplied by the number of reactions in the experiment, and distributed into the PCR tubes. Template and primers were added to individual tubes as dictated by the experiment setup. After PCR, 5 µl of PCR product mixed with 1 µl Massruler loading dye for each reaction was loaded onto separate wells on a 1% agarose gel, next to 5 µl Fastruler low range DNA ladder. Elctrophoresis was performed at 100V for 30 minutes, and the resulting bands were visualized using Syngene GeneGenius BIO imaging system.

Fecal DNA samples were refrigerated and stored in two film-sealed plates, distributed as shown in appendix 2, tables 18 and 19. For each sample, three PCR reactions were performed, one for each of *mdh* and *trpA*, using the same numbers for the forward and reverse primers for both per sample, and one control reaction with the 16S rRNA gene primers to confirm that the sample contained bacterial DNA of sufficient quality.

On plate 1, amplification was attempted for all samples, distributed in six batches of 14 samples and one batch of 12 samples, using *mdh* and *trpA* primers as indicated in appendix 2, table 20. Each batch had one positive control and one negative control for each of the three

types of primer. The primers for the controls were *mdh* F1R1, *trpA* F1R1, and 16S 515F 806R. Negative controls had no template, and positive controls used the P1 fecal DNA as a template.

The last of these batches also included two mock-samples, the first one using just ECOR34 DNA as template, the second one using a 50/50 mix of ECOR34 and ECOR42 DNA as template. These were made to help estimate the degree to which sequencing results would indicate the relative abundance of different strains within a sample.

In order to determine how well the samples covered the time period of the study, the number of successful amplifications for *mdh* and *trpA* were counted and visualized in figures 3 and 4. Full results of the sample amplifications can be found in appendix 2, table 22.

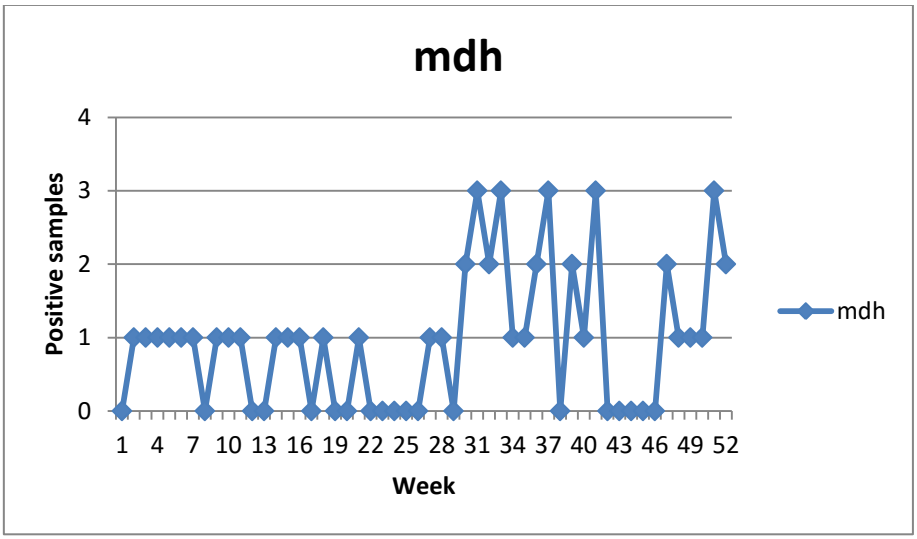



Figure 3. Distribution of samples from which *mdh* fragments were successfully amplified over the weeks of the study.

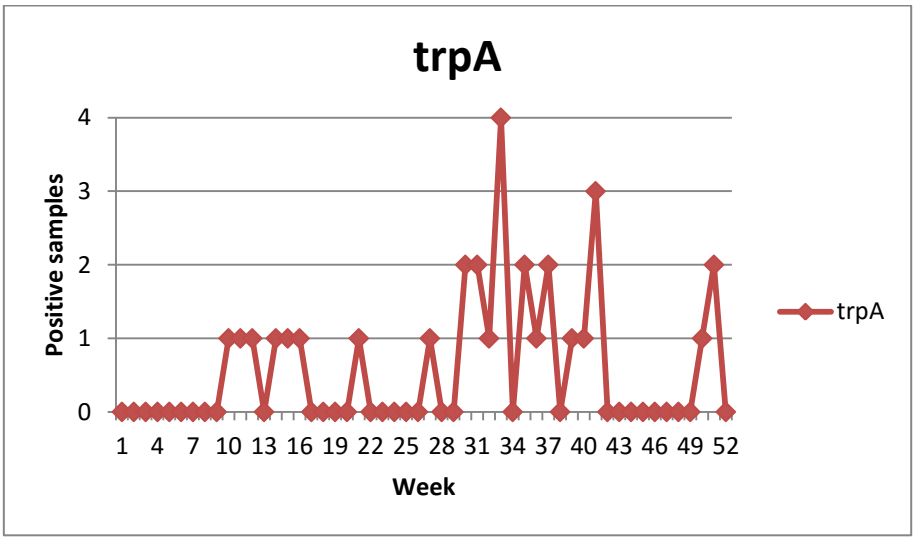



Figure 4. Distribution of samples from which *trpA* fragments were successfully amplified over the weeks of the study.

Based on this mapping, nine samples were picked from plate 2, from days not within the weeks covered by the successfully amplified samples from plate 1, and amplified using the same scheme as the batches described above. Sample IDs and primers used are found in appendix 2, table 21. Amplification was reattempted for samples where only one gene had been successfully amplified. Final set of samples to be included in the sequencing pool is shown in table 7.

| Sample day | *mdh* | *trpA* | Sample day | *mdh* | *trpA* |
|---|---|---|---|---|---|
| 9 | ✓ | ✓ | 230 | ✓ | ✓ |
| 18 | ✓ | | 237 | ✓ | |
| 26 | ✓ | | 239 | ✓ | ✓ |
| 31 | ✓ | ✓ | 244 | ✓ | ✓ |
| 41 | ✓ | | 247 | ✓ | ✓ |
| 45 | ✓ | | 256 | ✓ | ✓ |
| 57 | ✓ | ✓ | 258 | ✓ | ✓ |
| 68 | ✓ | ✓ | 267 | ✓ | ✓ |
| 74 | ✓ | ✓ | 270 | ✓ | |
| 79 | ✓ | ✓ | 280 | ✓ | ✓ |
| 96 | ✓ | ✓ | 284 | ✓ | ✓ |
| 105 | ✓ | ✓ | 287 | ✓ | ✓ |
| 112 | ✓ | ✓ | 328 | ✓ | ✓ |
| 126 | ✓ | | 329 | ✓ | ✓ |
| 143 | ✓ | ✓ | 334 | ✓ | ✓ |
| 187 | ✓ | ✓ | 337 | ✓ | ✓ |
| 196 | ✓ | ✓ | 349 | ✓ | ✓ |
| 209 | ✓ | ✓ | 351 | ✓ | ✓ |
| 214 | ✓ | | 357 | ✓ | ✓ |
| 215 | ✓ | ✓ | 362 | ✓ | |
| 218 | ✓ | ✓ | Custom sample 1 | ✓ | ✓ |
| 223 | ✓ | ✓ | Custom sample 2 | ✓ | ✓ |

Table 7. Final set of samples to be included in the sequencing pool

## 2.4 Pooling and purification

In order to prepare for sequencing, the selected samples had to be pooled together in volumes according to their relative DNA concentrations, to ensure that each sample would be equally represented in the sequencing data. The resulting sample pool then had to be purified to remove contaminants that might interfere with sequencing.

DNA concentrations in the selected samples were measured using a Qubit 2.0 Fluorometer with the Qubit double stranded DNA Broad Range assay kit, as described in the manual (Thermo Fisher Scientific 2015).

For all readings, sample assay tubes were prepared with 2µl sample and 198µl Qubit working solution.

The optimal total amount of DNA in the purified sequencing pool for the sequencing reaction was 1000ng, and it was estimated that about half the DNA would be lost during purification. As such, the desired amount of DNA from each of the 78 samples before purification would be 2000ng/78 ≈ 25ng.

Table 8 shows the calculated DNA concentration for each sample, as well as the volume added to the sequencing pool. For samples where the desired volume was lower than 1 µl, values are represented as fractions where the numerator indicates the volume added and the denominator indicates the degree of dilution with milliQ H2O.

| Sample day | *mdh* | | *trpA* | | Sample day | *mdh* | | *trpA* | |
|---|---|---|---|---|---|---|---|---|---|
| | Cons ng/µl | Volume µl | Cons ng/µl | Volume µl | | Cons ng/µl | Volume µl | Cons ng/µl | Volume µl |
| 9 | 53.3 | 1/2 | 9.38 | 2.5 | 230 | 18.5 | 1.5 | 31.3 | 1 |
| 18 | 3.52 | 7 | - | - | 237 | 7.76 | 3 | - | - |
| 26 | 20.7 | 1 | - | - | 239 | 43.0 | 3/5 | 62.3 | 2/5 |
| 31 | 5.16 | 5 | 6.02 | 4 | 244 | 19.1 | 1.5 | 12.9 | 2 |
| 41 | 27.3 | 1 | - | - | 247 | 91.2 | 2/7 | 35.2 | 3/4 |
| 45 | 47.3 | 1/2 | - | - | 256 | 8.71 | 3 | 27.3 | 1 |
| 57 | 105 | 1/4 | 12 | 2 | 258 | 161 | 1/6 | 29.9 | 1 |
| 68 | 4.57 | 5.5 | 8.17 | 3 | 267 | 39.8 | 3/5 | 18.4 | 1.5 |
| 74 | 18.1 | 1.5 | 10.1 | 2.5 | 270 | 60.7 | 4 | - | - |
| 79 | 11.9 | 2 | 26.8 | 1 | 280 | 28.4 | 1 | 56.2 | 1/2 |
| 96 | 3.06 | 8 | 4.4 | 5.5 | 284 | 37.5 | 2/3 | 12.6 | 2 |
| 105 | 3.11 | 8 | 3.5 | 7 | 287 | 6.95 | 4 | 15.6 | 1.5 |
| 112 | 4.12 | 6 | 18.8 | 1.5 | 328 | - | - | 53.6 | 1/2 |
| 126 | 5.05 | 5 | - | - | 329 | 10.1 | 2.5 | 13.3 | 2 |
| 143 | 26.5 | 1 | 34.2 | 3/4 | 334 | 8.34 | 3 | 13.7 | 2 |
| 187 | 9.33 | 3 | 29.4 | 1 | 337 | 17.2 | 1.5 | 16.2 | 1.5 |
| 196 | 13.4 | 2 | 13.9 | 2 | 349 | 14.6 | 2 | 17.3 | 1.5 |
| 209 | 36.1 | 7 | 24.7 | 1 | 351 | 6.43 | 4 | 16.5 | 1.5 |
| 214 | 19.6 | 1.5 | - | - | 357 | 39.4 | 2/3 | 101 | 1/4 |
| 215 | 8.35 | 3 | 20 | 1 | 362 | 4.02 | 6 | - | - |
| 218 | 53.1 | 1/2 | 19.1 | 1.5 | Custom sample 1 | 110 | 1/4 | 236 | 1/10 |
| 223 | 51.9 | 1/2 | 59.2 | 2/5 | Custom sample 2 | 163 | 1/6 | 173 | 1/6 |

Table 8. Concentration and volume added for all samples in the sequencing pool. Samples marked in red were added in tenfold higher volumes than intended due to a calculation error.

The pooled samples were purified using the QIAquick PCR Purification kit, as described in the manual using the microcentrifuge protocol (Qiagen 2010). Elution was performed using MiliQ $H_2O$.

After purification, 5 µl of the sequencing pool was mixed with 1 µl Massruler loading dye and loaded onto a 1% agarose gel, next to 5 µl Fastruler low range DNA ladder. Electrophoresis was performed at 100V for 30 minutes, and the resulting bands were visualized using the Syngene GeneGenius BIO imaging system.. (Shown in appendix 3, figure 25.) As the visualization displays two distinct bands in the expected size ranges for *mdh* and *trpA*, the sample pool was cleared for sequencing. 1µl was used to measure the DNA concentration using a NanoDrop spectrophotometer and was found to be 24,4ng/µl.

## 2.5 Sequencing

44µl of the purified pooled samples, with estimated total DNA content of 1074ng, was submitted for Single molecule real time sequencing on a Pacific Biosciences RS II sequencer using a single SMRT cell.

The sequencing service was provided by the Norwegian Sequencing Centre (www.sequencing.uio.no), a national technology platform hosted by the University of Oslo and supported by the "Functional Genomics" and "Infrastructure" programs of the Research Council of Norway and the Southeastern Regional Health Authorities.

Results were filtered by quality, and two fastq files were generated as output, one with a quality cut-off of 90% accuracy, and one with a quality cut-off of 99% accuracy. Full sequencing report can be found in appendix 4.

### 2.5.1 Filtering sequencing results

In order to separate the reads from the sequencing results by source sample, and to count the number of identical reads within an individual sample, two workflows were made in Lifeportal, a UiO maintained install of Galaxy running on the Abel high performance computing cluster. Full workflows can be found at https://lifeportal.uio.no/u/sigmunr%40uio.no/w/filtering-ecoli-pool-by-primer-sequences-*mdh* and https://lifeportal.uio.no/u/sigmunr%40uio.no/w/filtering-ecoli-pool-by-primer-sequences-*trpa*,

and a schematic representation of the demultiplexing process is shown in figure 5.

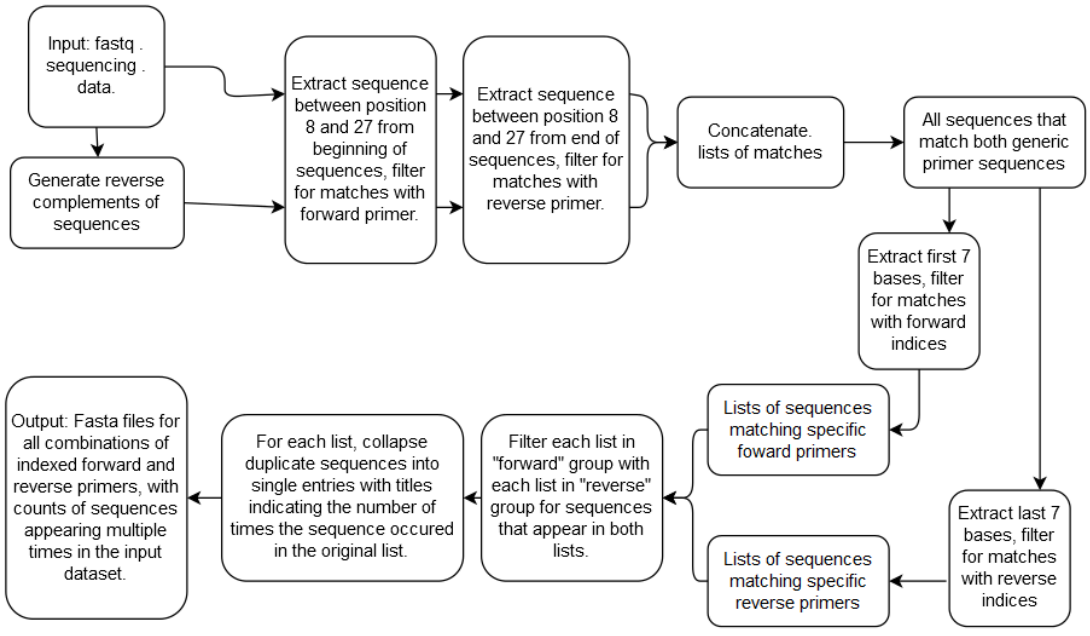



Figure 5. Schematic representation of the demultiplexing process performed in the Lifeportal workflows.

Because Lifeportal was not up to date with the development version of Galaxy when these workflows were designed, they were not able to benefit from new features that allow for more simple iteration over large numbers of datasets, such as Dataset Collections or Multiple File Datasets. Because of this the workflows are quite unwieldy, and cannot easily be modified to filter out other combinations of primers, or to filter by different primers or indices. Although they can be used for technical replication of the analysis process, it is recommended that future experiments create workflows on an updated version of Galaxy, use a different platform altogether, or use existing demultiplexing pipelines.

Tools used in the workflow:

FastQ to FastA (v1.0.0)(Blankenberg et al. 2010), Revseq (6.5.7)(Blankenberg et al. 2007), Collapse (0.0.13), Tabular-To-FASTA, FASTA-To-Tabular, Cut, Trim, Compare, Filter.

# 3 Results and discussion

## 3.1 Sample coverage

Fecal samples were collected by the subject's parents at semi-regular intervals over a period of 365 days, or just over 52 weeks, starting with the the subject's date of birth. Although the samples were only taken on 35,9% of the days during the year of the study, they were distributed in such a way that there was at least one sample taken in 82,7% of the weeks in the trial period. (Distribution of samples taken and sequenced over days and weeks shown in table 9)

| Category | Nr. of days | % of days | Nr. of weeks | % of weeks |
|---|---|---|---|---|
| Not sampled | 234 | 64,1 | 9 | 17,3 |
| Sampled but not sequenced | 90 | 24,7 | 9 | 17,3 |
| Sampled and sequenced for only one gene | 10 | 2,7 | 7 | 13,5 |
| Sampled and sequenced for both genes | 32 | 8,8 | 27 | 51,9 |

Table 9. Distribution of sample coverage over the days and weeks of the study period.

The nine weeks where no samples were taken were nr. 13, nr. 22-25, and nr. 43-46, the latter two sets of weeks accounting for the two largest gaps in the resulting dataset. (A map of the week by week sample coverage can be seen in figure 6.)

Additionally, weeks 17-20 only had one sample for *mdh* and none for *trpA* that were successfully amplified and sequenced, which might be indicative of the *E. coli* DNA concentration in the samples in this time period being below or close to the amplification limit for the selected primers, or the samples contained some form of contaminant that interfered with amplification. All samples within this time period were attempted amplified in separate reactions on different days, and for all of them some of the other amplification reactions performed the same day using the same reaction mixture and conditions were successful, indicating that these failed amplifications were likely not caused by systematic errors during amplification, but rather due to the properties of these particular samples.

Lastly, one sample, *trpA* day 230, was added to the sequencing pool, but no reads were identified after demultiplexing. This might result from accidentally applying the incorrect primers to the reaction mix during amplification, or from an error during the application of the sample to the sequencing pool.

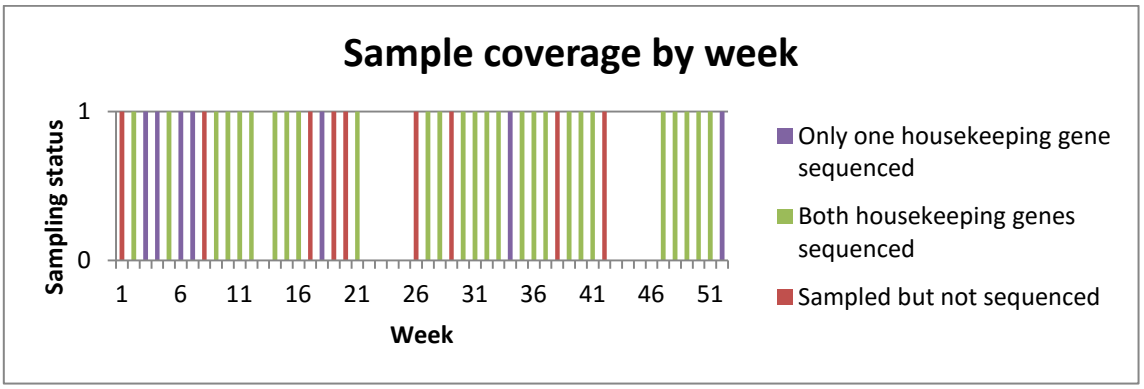



Figure 6. Distribution of samples and successful amplifications over the weeks of the study period.

## 3.2 Identifying strains

In order to reduce the interference of spurious sequences in the dataset, sequences that appeared fewer than three times in a particular sample were not included in the analysis. The remaining sequences were labelled by searching for the closest matching named allele in the Shigatox and Pasteur MLST databases for *mdh* and *trpA* respectively. In order to test the validity of this naming scheme, and to compare the read number and signal to noise ratio of the 90% accuracy cut-off and 99% accuracy cut-off datasets, the alleles present for both genes were first identified in the synthetic control samples, whose templates contained just reference strain ECOR34 DNA or a 50/50 mix of ECOR34 and ECOR42 DNA.

Based on the MLST data for the ECOR reference strains in the Shigatox and Pasteur MLST databases, the expected alleles for ECOR34 were *mdh*8 and *trpA*8, and for ECOR42 were *mdh*130 and *trpA*36. For both datasets, looking at the sequences with frequencies above the cut-off limit, only the expected alleles were present in the sequencing data for each sample (figures 7 and 8), but the samples with mixed templates heavily favoured the ECOR42 sequences. This indicated that ECOR42 was present at a higher relative frequency than ECOR34. The difference in the number of sequences that appeared more than three times was negligible between the two datasets, while there was a slight increase in the number of sequences that appeared three or fewer times in the dataset using 90% accuracy as the cut-off in the quality filtering, compared to the dataset using 99% accuracy as the cut-off, leading to a slightly lower noise to signal ratio (table 10). Because of this, the 99% accuracy cut-off dataset was used in all further analysis.

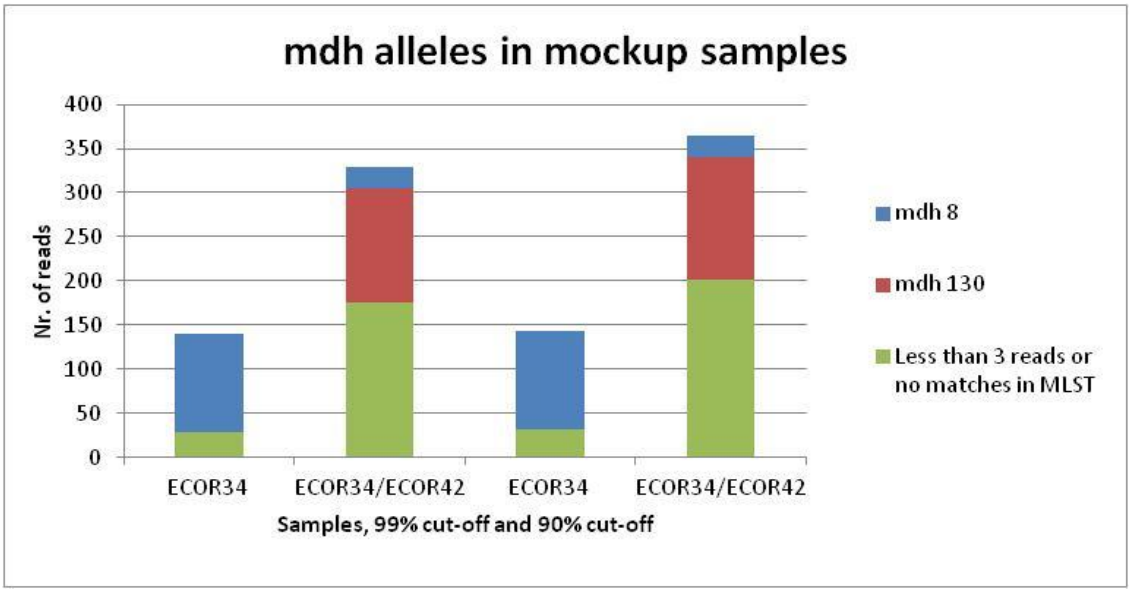



Figure 7. Distribution of identified sequences in the synthetic *mdh* control samples using different levels of quality filtering.

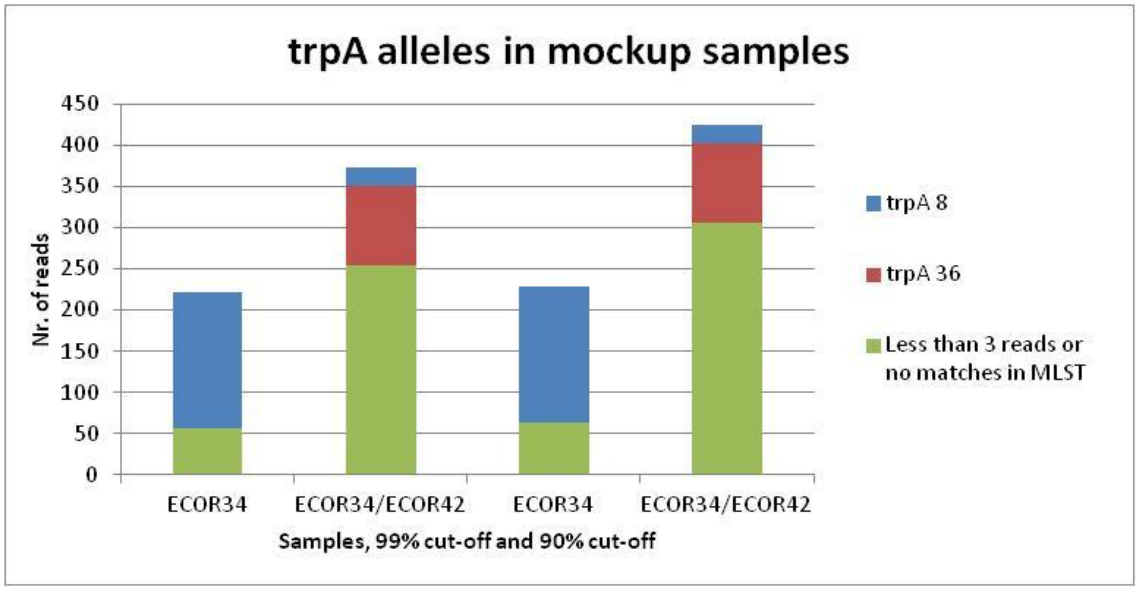



Figure 8. Distribution of identified sequences in the synthetic *trpA* control samples using different levels of quality filtering.

| Samples | Identified sequences | Discarded sequences | Signal to noise ratio |
|---|---|---|---|
| *mdh*, 99% accuracy | 266 | 203 | 1,31 |
| *trpA*, 99% accuracy | 283 | 312 | 0,91 |
| *mdh*, 90% accuracy | 275 | 233 | 1,18 |
| *trpA*, 90% accuracy | 284 | 369 | 0,77 |

Table 10. Signal to noise ratios for the synthetic samples under different levels of quality filtering.

Following this, sequences from all samples in the dataset were compared against the named alleles in the Shigatox and Pasteur MLST databases, and the following alleles, or close relatives thereof, were identified (table 11). For all sequences examined, there were either found exact matches in the MLST databases, or closely resembled sequences with exact matches that appeared more frequently in the same samples, suggesting that these represented minor amplification or sequencing errors, rather than novel alleles.

| *mdh* alleles | *trpA* alleles |
|---|---|
| *mdh* 1 | *trpA* 1 |
| *mdh* 2 | *trpA* 2 |
| *mdh* 5 | *trpA* 8 |
| *mdh* 8 | *trpA* 10 |
| *mdh* 35 | *trpA* 12 |
| *mdh* 36 | *trpA* 19 |
| *mdh* 60 | *trpA* 36 |
| *mdh* 85 | *trpA* 139 |
| *mdh* 96 | |
| *mdh* 122 | |
| *mdh* 130 | |

Table 11. Closest resembling alleles in MLST databases to sequence variants appearing in sequencing data.

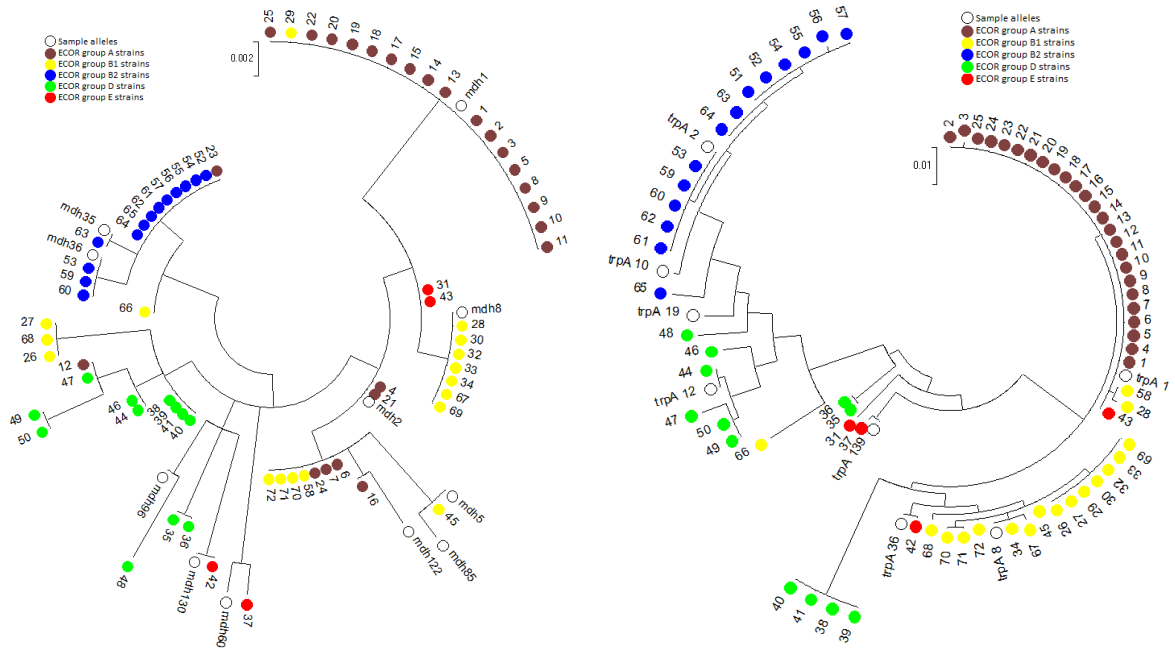
In order to confirm if all the identified sequences were representative of different strains, pairwise distance matrices were generated for both the *mdh* alleles and the *trpA* alleles using the Maximum Composite Likelihood method in MEGA 7.0.14 (Appendix 2, table 23 and 24). If two allele sequences have a very high degree of similarity, are found predominantly or exclusively in the same samples, and one has a lower frequency than the other, this would be indicative of one of the sequences possibly being the result of misreads of the other during sequencing, rather than coming from separate strains.

For the *mdh* alleles, the pairs displaying a very high degree of similarity were *mdh*2-*mdh*8, *mdh*2-*mdh*122, and *mdh*35-*mdh*36. For the *trpA* alleles, the only pair displaying a very high degree of similarity was *trpA*2-*trpA*10. For each of these pairs the number of samples each allele was found in, and the number of samples where they appear together are listed in table 12. (Full table of alleles found for each sample can be found in appendix 2, tables 25 and 26). Since both *mdh*2 and *mdh*8 both appear in multiple separate samples, it is safe to conclude that these two alleles represent (at least) two different strains that are present in the dataset. *mdh*122 and *trpA*10 may represent misreads of *mdh*2 and *trpA*2 respectively, but since the number of reads for each are not very different within each sample, all four alleles were retained as separate in further analysis. For *mdh*35 and *mdh*36, some of the reads in the samples where both occur may result from sequencing errors, however, when comparing the relative abundance of reads between the two alleles for each sample, it's found that each allele is dominant in a different stretch of the trial period. (Days 196 to 230 for *mdh*36, and days 247 to 284 for *mdh*35). This suggests that the alleles represent (at least) two different strains present in the dataset, and both are retained as separate for further analysis.

| Allele pair | Nr. Samples with first allele | Nr. samples with second allele | Nr. samples with both alleles |
|---|---|---|---|
| *mdh*2<br>*mdh*8 | 8 | 7 | 1 |
| *mdh*2<br>*mdh*122 | 8 | 2 | 2 |
| *Mdh*35<br>*mdh*36 | 12 | 11 | 7 |
| *trpA*2<br>*trpA*10 | 6 | 2 | 1 |

Table.12. Overlap and lack thereof for sequences with a high degree of similarity.

*E. coli* strains are commonly divided into five phylogenetic groups: A, B1, B2, D, and E (Carlos et al. 2010). In order to better characterize the different sequences found in the sequencing data, phylogenetic groups were assigned to the alleles using a method based on previous work by Eric de Muinck (de Muinck, Øien et al. 2011). Using the *mdh* and *trpA* sequences from the Shigatox and Pasteur MLST databases for all ECOR reference strains to provide a phylogenetic framework, (with the exception of ECOR51 *mdh*, which was not represented by an isolate in the Shigatox database,) phylogenetic trees were generated with the sample alleles for both *mdh* and *trpA* by Maximum Likelihood using MEGA 7.0.14 (Figures 9 and 10). As expected based on the results of the previous study, sequences divide broadly into the expected phylogenetic groups, but with a number of misassigned sequences, due to loss of information in single gene typing versus multi gene typing. Because of this, and due to placement of sample alleles between the established phylogenetic groups in some cases, there is some ambiguity in the assignment of phylogenetic groups for some alleles. Assigned phylogenetic groups for all alleles can be found in table 13.



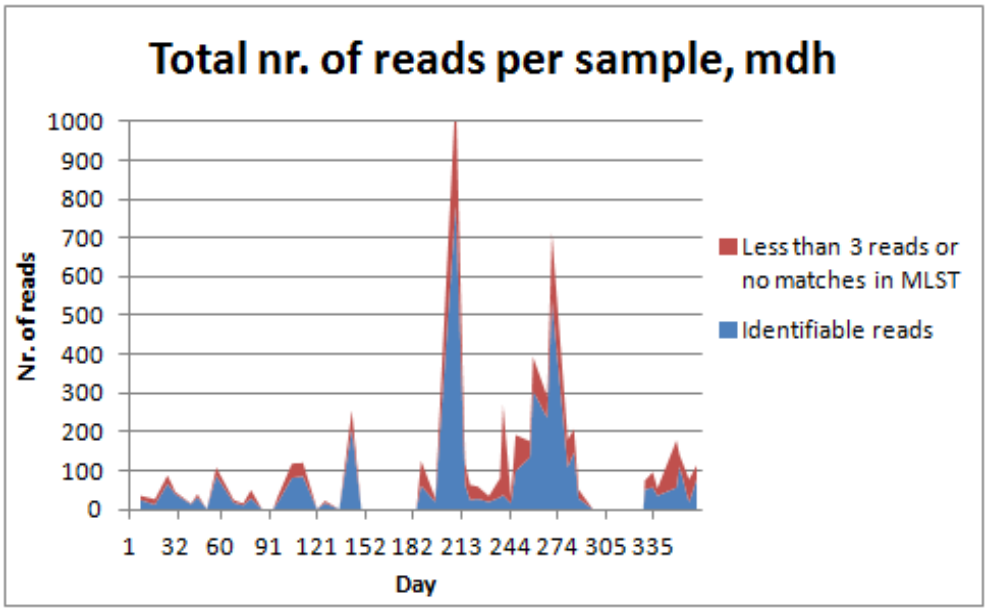

Figures 9 and 10. Phylogenetic analysis of *mdh* and *trpA* strains. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The trees with the highest log likelihoods are shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Tamura et al. 1993, Kumar et al. 2016).

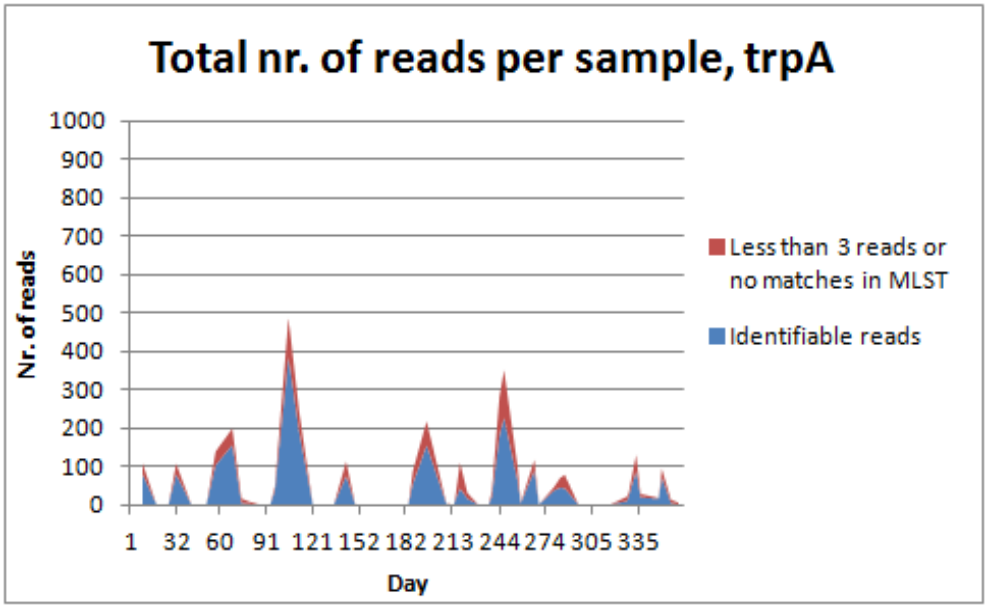
| *mdh* alleles | Phylogenetic group | *trpA* alleles | Phylogenetic group |
|---|---|---|---|
| *mdh* 1 | A | *trpA* 1 | A |
| *mdh* 2 | A or B1 | *trpA* 2 | B2 |
| *mdh* 5 | B1 | *trpA* 8 | B1 |
| *mdh* 8 | B1 | *trpA* 10 | B2 |
| *mdh* 35 | B2 | *trpA* 12 | D |
| *mdh* 36 | B2 | *trpA* 19 | B2 or D |
| *mdh* 60 | E | *trpA* 36 | B1 or E |
| *mdh* 85 | B1 | *trpA* 139 | E |
| *mdh* 96 | D | | |
| *mdh* 122 | A or B1 | | |
| *mdh* 130 | E | | |

Table 13. Assigned phylogenetic groups for all identified alleles in the sequencing data.

## 3.3 Mapping strain distribution

For the majority of the samples for both *mdh* and *trpA*, the total numbers of sequencing reads per sample was somewhere below 500 reads, with two major exceptions; *mdh* day 209, with 1075 reads, and *mdh* day 270, with 712 reads. As noted in the Pooling and Purification section, these two samples were added to the sequencing pool in tenfold higher volumes than intended, due to a calculation error. These two samples alone account for respectively 11,4% and 7,6% of the 9425 reads that could successfully be traced back to specific samples. In the ideal case, where each sample was represented equally in the sequencing data, the expected value would be 1,3%, or roughly 120 reads per sample.




Figures 11 and 12. Mapping of read numbers per day over the study period for both *mdh* and *trpA* datasets.

The distribution of alleles identified per sample over the year of the sample is shown in figure 13 for *mdh*, and figure 14 for *trpA*. (Read numbers for all samples can be found in appendix 2, tables 25 and 26). The strain composition can be divided into five blocks of relative stability, beginning and ending with short transitional periods with higher strain diversity, or during periods with no sampling data:

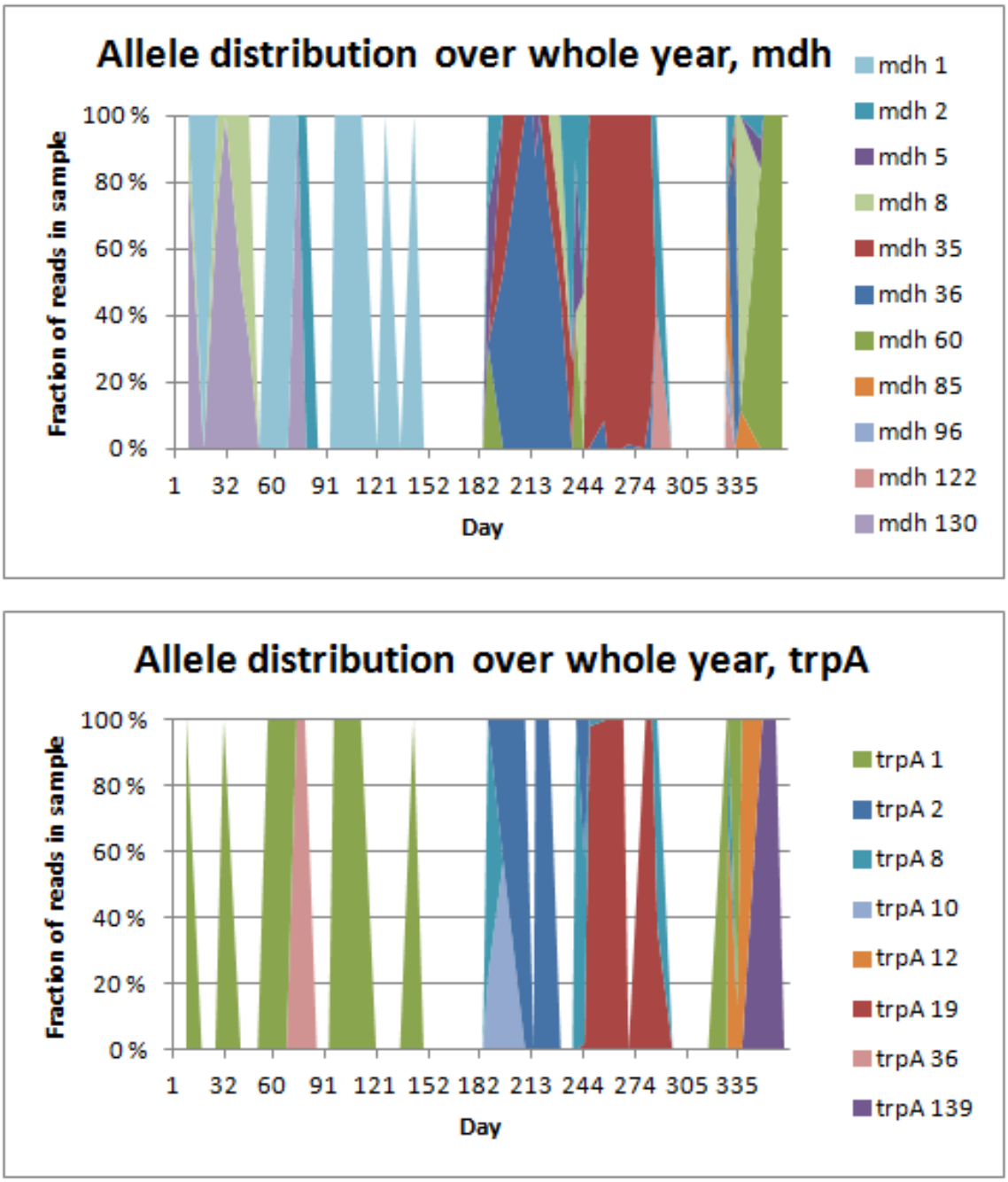
1. Days 9-79: During this period, the alleles found in *mdh* samples fluctuates between *mdh*1, or *mdh*8 and *mdh*130 coexisting. *trpA* coverage is scarce during this early period, but the only allele identified in most of the samples in this period was *trpA*1. The end of this first block is marked by the sudden appearance of *mdh*2 and *trpA*36, and the first week with no samples taken.

2. Days 96-143: During the entirety of this period, only one allele was detected for both *mdh* and *trpA*: *mdh*1 and *trpA*1. This continues to the end of the block, which is marked by the first of the two month long periods during which no samples were taken.

3. Days 187-244: At the beginning of this period on day 187, the following alleles were identified: *mdh*2, *mdh*5, *mdh*60 *trpA*8 and *trpA*10. From day 196 to 230, the dominant alleles found were *mdh*36 and *trpA*2, with sporadic appearances of *mdh*5, *mdh*8, and *mdh*35. At the end of the block, the dominant allele was replaced by a mix of *mdh*2, *mdh*5, *mdh*8, *mdh*36, *mdh*60, and *trpA*80.

4. Days 247-287: Following the transitional period at the end of the previous block, the dominant alleles found in this block were *mdh*35 and *trpA*19, with sporadic appearances of *mdh*36 and *trpA*8. At the end of the block, the dominant allele was replaced by *mdh*2, *mdh*122, *trpA*1 and *trpA*8, followed by the second month long period during which no samples were taken.

5. Days 329-362: At the beginning of this block, between day 329 and 337, a large number of different alleles were identified: *mdh*2, *mdh*5, *mdh*8 *mdh*35, *mdh*36, *mdh*85, *mdh*96, *mdh*122, *trpA*1, *trpA*8, and *trpA*12. From day 349 to the end of the study period, the dominant alleles were *mdh*60 and *trpA*139.




Figures 13 and 14. Mapping of allele distribution per day over the study period for both *mdh* and *trpA* datasets.

The four blocks where single *mdh* and *trpA* alleles were identified, were postulated to represent single strains, or a number of very closely related strains, which were designated with the letters A to D as shown in table 14.

| Designation | *mdh* allele | Time period | *trpA* allele | Time period |
|---|---|---|---|---|
| A | *mdh* 1 | Day 96-143 | *trpA* 1 | Day 96-143 |
| B | *mdh* 36 | Day 196-230 | *trpA* 2 | Day 196-223 |
| C | *mdh* 35 | Day 247-284 | *trpA* 19 | Day 247-284 |
| D | *mdh* 60 | Day 349-362 | *trpA* 139 | Day 349-357 |

Table14. Designations of suspected dominant strains present in sequencing data for both genes.

In order to categorize these strains into phylogenetic groups, concatenated sequences were generated for all ECOR reference strains except ECOR51, by attaching the sequences of their *trpA* alleles to their *mdh* alleles from the Shigatox and Pasteur databases, head to tail. These were used to provide a phylogenetic framework, and the concatenated sequences of the sample strains were mapped onto a phylogenetic tree generated by Maximum Likelihood using MEGA 7.0.14 (Figure 15). The tree generated using the sequences of both genes together matched the expected distribution of ECOR strains into the five phylogenetic groups better than either of the trees generated using one of the genes alone (figures 9 and 10), with only five strains not falling neatly into the expected distributions (ECOR28, ECOR42, ECOR 43, ECOR58, and ECOR66).

Sample strains were categorized by their position in the phylogenetic tree, and categorization matched with the one performed single genes for both *mdh* and *trpA* (Table 15).

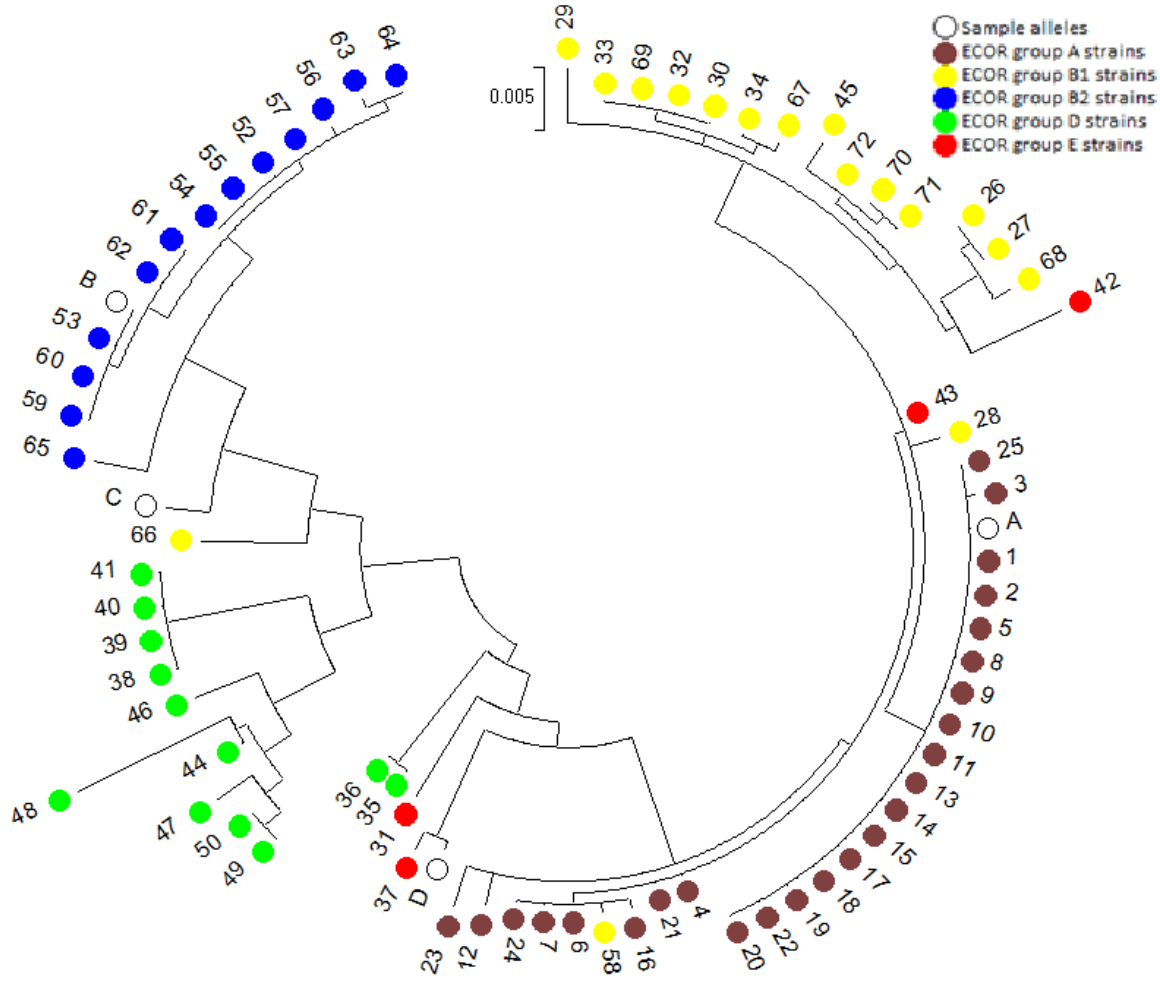



Figure 15. Phylogenetic analysis of combined MLST data for *trpA* and *mdh*. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-3105.2307) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with

superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 75 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1110 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura and Nei 1993, Kumar, Stecher et al. 2016).

| Designation | Phylogenetic group predicted with both *mdh* and *trpA* | Phylogenetic group predicted with *mdh* | Phylogenetic group predicted with *trpA* |
|---|---|---|---|
| A | A | A | A |
| B | B2 | B2 | B2 |
| C | B2 or D | B2 | B2 or D |
| D | E | E | E |

Table15.Comparison of phylogenetic group assignment between combined gene and single-gene phylogenetic analysis.

## 3.4 Metadata and environmental factors

In order to protect the identity of the subject and keep the study blind, the parents of the subject were questioned about significant events, travel, antibiotics use and sickness, using Eric de Muinck as a proxy to anonymize the data. The parents were also asked to make note of events around the dates marking the beginning and end of the blocks identified in the section Mapping Strain Distribution: Days 79, 96, 143, 187, 244, 287, and 329.

The subject was vaccinated on days 99, 200, and 362. All of these days fall within the stable sections of blocks with single dominant strains, after the strain has been established, and do not seem to have any immediate effect on strain composition.

Neither the subject not their mother used antibiotics during the year of the study, and no instances of disease were noted by the parents.

The subject's diet began to include solid foods sometimes around days 88-95, which matches with the transition between blocks 1 and 2, and the transition may be caused by this change in diet.

It was noted that the longer time periods in which no samples were taken occurred as a result of travel during the study year. Both blocks 3 and 5 follow directly from extended time periods without sampling, and begin with short periods of high strain diversity, before it stabilizes into the dominant strains.

While these observations suggest, as expected, that major changes in the gut microbiome composition generally happen as a result of changes in environmental factors such as diet and travel, it is difficult to make more detailed conclusions or predictions without having similarly detailed datasets for multiple subjects to compare.

## 3.5 Strain properties

Of the five phylogenetic groups of *E. coli* A and B2 are the ones most commonly found in the human microbiome by global average distribution, appearing as dominant in 40.5% and 25.5% of cases respectively.(Tenaillon, Skurnik et al. 2010) However, the exact rates of dominance for reach group varies from country to country, and previous studies on Norwegian and Swedish infants suggest that B2 is the predominant early colonizer of the infant gut microbiome in these populations, to which the subject belongs. In spite of this, the alleles found in samples from the early microbiome of the subject belonged predominantly to group A, along with B1 and E, and B2 did not become the dominant strain until approximately half a year into the study.

Although phylogenetic group E is known to contain enterohaemorragic strains, (such as many of the O157:H7 serotype strains,) the link between phylogenetic distance and pathology is small(Gordon et al. 2003), and no disease was noted by the parents during block 5, where a group E strain was dominant. Since previous studies suggest that group E strain dominance is rare in early colonization of Scandinavian infants, (0.4% of infants in the 2011 study) (de Muinck, Øien et al. 2011), its presence in block 5 gives further credence to the idea that the last observed shift in the population structure of the subject's microbiome resulted from changes in environment during travel.

## 3.6 Scalability of experimental design

In order to adapt the methodology described in this paper to a larger study comparing the microbiome of multiple infants, it would be necessary to amplify and sequence a much larger number of samples (for ideal coverage of one sample per week for a whole year, 52 samples per gene amplified per infant). The most expensive processing step in this procedure is the SMRT sequencing, and as such it would be desirable to have the largest number of samples per sequencing reaction that would still produce viable data.

Using the exact primers designed for this project, the largest number of distinct samples that could theoretically be sequenced in the same reaction would be 140 per gene (14 distinct forward primers and 10 distinct reverse primers). When generating index sequences using the script described in appendix 1, it is possible to generate 64 distinct index sequences 5 nucleotides long where each is at least three bases different from every other in the set, which were the same parameters used while picking sequences for this project. If no indices are reused between reverse and forward primers, this allows for a maximum of 1024 combinations without reducing the difference threshold or increasing sequence length while designing index sequences. As such, primer design should not be considered a limiting factor in multiplexing.

According to the sequencing report, the one SMRT cell used in this project returned 46842 reads, of which 26290 (56%) passed 0.99 minimum accuracy filtering. Of these, 9425 could be assigned to specific samples, and 6307 were deemed to be of sufficient quality to be used in strain categorization. If these rates are assumed to be representative for an average run using this methodology, the average expected number of usable reads per sample if running 104 samples (52 per gene for two genes) on a single SMRT cell is $6307/104 \approx 60$. Because of this, unless the signal to noise ratio is significantly improved, it is not recommended to sequence sample sets from more than one subject per SMRT cell, and higher sample volumes have to be handled by using higher numbers of SMRT cells per sequencing run.

# 4 Conclusion

The methodology described and tested in this thesis allows for the mapping of the dynamics of the developing infant gastrointestinal microbiome at a much higher resolution than previous studies, and is suitable for use in future studies comparing the microbiomes of multiple infants, though further optimization is desirable to reduce the amount of malformed or junk reads. This increased resolution will potentially allow for the tracing of changes in the gut microbiome to specific environmental factors, and provide greater understanding of the normal development of the neonatal microbiome in healthy infants, and how changes in strain composition occurs. Using multiple genes from different MLST schemes allows for more accurate classification of ambiguous strains, although this is limited to dominant strains unless pure cultures are generated from the sample material.

The study was able to sample 43 of the 52 weeks of the study period, determine five timespans with different dominant *E. coli* strains, identify potential environmental factors relating to travel and changes in diet that might be linked to the changes between these periods, and classify multiple competing strains during periods where the strain composition of the gut microbiome is undergoing changes. Alleles suggesting the presence of at least 11 different strains colonizing the gut microbiome of the subject during the study period were identified. This is a larger number than similar previous studies, and can be explained by the much higher sample coverage.

# 5 Appendix

### Appendix 1: Primer-index generating script

```
print "Length of index sequences?"
indexlength = raw_input()
inxlen = int(indexlength)
print "Minimum nr. of different bases?"
difference = raw_input()
diff = inxlen - int(difference)
indexlist=[]
bases="ATGC"
testlist = ["A","T","G","C"]
progress = 2
while progress <= inxlen:
    templist = []
    for seq in testlist:
        for base in bases:
            templist.append(seq+base)
    testlist = templist
    progress = progress + 1
for teststring in testlist:
    print "Checking " + teststring
    maxsimilarity = 0
    for index in indexlist:
        pos = 0
        similarity = 0
        while pos <= inxlen-1:
            if index[pos] == teststring[pos]:
                similarity = similarity + 1
            pos = pos + 1
        if similarity > maxsimilarity:
            maxsimilarity = similarity
    if maxsimilarity > diff:
        print "Too similar"
    else:
        print "Added"
        indexlist.append(teststring)
print "Final list:"
print indexlist
length1 = len(indexlist)
length2 = str(length1)
print length2 + " indexes found."
saveq = 0
while saveq == 0:
    saveoutput = raw_input ("Save output to file? y/n: ")
    if "y" in saveoutput.lower():
        filename = raw_input ("File name? ") +".txt"
        fo = open(filename, "w")
        for index in indexlist:
            fo.write ("%s\n" % index)
        fo.close()
        saveq = 1
    elif "n" in saveoutput.lower():
        saveq = 1
```

## Appendix 2: Miscellaneous tables

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| AAAAA | AATTT | AAGGG | AACCC | ATATG | ATTAC | ATGCA | ATCGT |
| AGAGC | AGTCG | AGGAT | AGCTA | ACACT | ACTGA | ACGTC | ACCAG |
| TAATC | TATAG | TAGCT | TACGA | TTAAT | TTTTA | TTGGC | TTCCG |
| TGACA | TGTGT | TGGTG | TGCAC | TCAGG | TCTCC | TCGAA | TCCTT |
| GAAGT | GATCA | GAGAC | GACTG | GTACC | GTTGG | GTGTT | GTCAA |
| GGAAG | GGTTC | GGGGA | GGCCT | GCATA | GCTAT | GCGCG | GCCGC |
| CAACG | CATGC | CAGTA | CACAT | CTAGA | CTTCT | CTGAG | CTCTC |
| CGATT | CGTAA | CGGCC | CGCGG | CCAAC | CCTTG | CCGGT | CCCCA |

Table 16. Index sequences generated during primer design.

| Primer name | Sequence | Primer name | Sequence | Primer name | Sequence | Primer name | Sequence |
|---|---|---|---|---|---|---|---|
| *MDH* fw1 | AAAAAGAG TCGATCTG AGCCATAT CCCTAC | *MDH* rv1 | ACACTGC TACTGAC CGTCGCC TTCAAC | TRPA fw1 | TTAATTG GCTACGA ATCTCTG TTTGCC | TRPA rv1 | GAAGTGG GCTTTCAT CGGTTGT ACAAA |
| *MDH* fw2 | AATTTGAG TCGATCTG AGCCATAT CCCTAC | *MDH* rv2 | ACTGAGC TACTGAC CGTCGCC TTCAAC | TRPA fw2 | TTTTATG GCTACGA ATCTCTG TTTGCC | TRPA rv2 | GATCAGG GCTTTCAT CGGTTGT ACAAA |
| *MDH* fw3 | AAGGGGAG TCGATCTG AGCCATAT CCCTAC | *MDH* rv3 | ACGTCGC TACTGAC CGTCGCC TTCAAC | TRPA fw3 | TTGGCTG GCTACGA ATCTCTG TTTGCC | TRPA rv3 | GAGACGG GCTTTCAT CGGTTGT ACAAA |
| *MDH* fw4 | AACCCGAG TCGATCTG AGCCATAT CCCTAC | *MDH* rv4 | ACCAGGC TACTGAC CGTCGCC TTCAAC | TRPA fw4 | TTCCGTG GCTACGA ATCTCTG TTTGCC | TRPA rv4 | GACTGGG GCTTTCAT CGGTTGT ACAAA |
| *MDH* fw5 | ATATGGAG TCGATCTG AGCCATAT CCCTAC | *MDH* rv5 | TAATCGC TACTGAC CGTCGCC TTCAAC | TRPA fw5 | TGACATG GCTACGA ATCTCTG TTTGCC | TRPA rv5 | GTACCGG GCTTTCAT CGGTTGT ACAAA |
| *MDH* fw6 | ATTACGAG TCGATCTG AGCCATAT CCCTAC | *MDH* rv6 | TATAGGC TACTGAC CGTCGCC TTCAAC | TRPA fw6 | TGTGTTG GCTACGA ATCTCTG TTTGCC | TRPA rv6 | GTTGGGG GCTTTCAT CGGTTGT ACAAA |
| *MDH* fw7 | ATGCAGAG TCGATCTG AGCCATAT CCCTAC | *MDH* rv7 | TAGCTGC TACTGAC CGTCGCC TTCAAC | TRPA fw7 | TGGTGTG GCTACGA ATCTCTG TTTGCC | TRPA rv7 | GTGTTGG GCTTTCAT CGGTTGT ACAAA |
| *MDH* fw8 | ATCGTGAG TCGATCTG AGCCATAT CCCTAC | *MDH* rv8 | TACGAGC TACTGAC CGTCGCC TTCAAC | TRPA fw8 | TGCACTG GCTACGA ATCTCTG TTTGCC | TRPA rv8 | GTCAAGG GCTTTCAT CGGTTGT ACAAA |
| *MDH* fw9 | AGAGCGAG TCGATCTG AGCCATAT CCCTAC | *MDH* rv9 | GGGGAGC TACTGAC CGTCGCC TTCAAC | TRPA fw9 | TGCACTG GCTACGA ATCTCTG TTTGCC | TRPA rv9 | GCGCGGG GCTTTCAT CGGTTGT ACAAA |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *MDH* fw10 | AGTCGGAG TCGATCTG AGCCATAT CCCTAC | *MDH* rv10 | GGCCTGC TACTGAC CGTCGCC TTCAAC | TRPA fw10 | TCTCCTG GCTACGA ATCTCTG TTTGCC | TRPA rv10 | GCCGCGG GCTTTCAT CGGTTGT ACAAA |
| *MDH* fw11 | AGGATGAG TCGATCTG AGCCATAT CCCTAC | | | TRPA fw11 | TCGAATG GCTACGA ATCTCTG TTTGCC | | |
| *MDH* fw12 | AGCTAGAG TCGATCTG AGCCATAT CCCTAC | | | TRPA fw12 | TCCTTTG GCTACGA ATCTCTG TTTGCC | | |
| *MDH* fw13 | GGAAGGAG TCGATCTG AGCCATAT CCCTAC | | | TRPA fw13 | GCATATG GCTACGA ATCTCTG TTTGCC | | |
| *MDH* fw14 | GGTTCGAG TCGATCTG AGCCATAT CCCTAC | | | TRPA fw14 | GCTATTG GCTACGA ATCTCTG TTTGCC | | |

Table 17. Sequences of all primers designed for this project

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | PC1 | 223 | 334 | 287 | 229 | 269 | PC2 | 361 | 359 | 262 | 12 | 74 |
| B | NC | 357 | 349 | 211 | 350 | 222 | 272 | 236 | 263 | 248 | 200 | 45 |
| C | 216 | 239 | 330 | 225 | 226 | 258 | 270 | 238 | 212 | 363 | 69 | 196 |
| D | 218 | 227 | 351 | 245 | 210 | 237 | 281 | 282 | 261 | 67 | 185 | 23 |
| E | 280 | 231 | 329 | 285 | 256 | 345 | 209 | 331 | 249 | 62 | 79 | 143 |
| F | 328 | 247 | 215 | 265 | 241 | 365 | 198 | 337 | 230 | 122 | 26 | 41 |
| G | 214 | 284 | 275 | 267 | 256 | 257 | 283 | 244 | 213 | 141 | 31 | 57 |
| H | 242 | 362 | 187 | 288 | 352 | 217 | 289 | 246 | 279 | 139 | 9 | PC3 |

Table 18. Plate 1 map, sampling days

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | PC1 | 112 | 14 | 76 | 182 | 105 |
| B | NC | 199 | 8 | 64 | 77 | 19 |
| C | 51 | 38 | 18 | 73 | 197 | 128 |
| D | 55 | 142 | 180 | 113 | 61 | |
| E | 191 | 188 | 1 | 192 | 21 | |
| F | 33 | 192 | 126 | 130 | 68 | |
| G | 16 | 75 | 10 | 131 | 35 | |
| H | 66 | 22 | 11 | 96 | 71 | |

Table 19. Plate 2 map, sampling days

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | F1 R1 | F9 R1 | F6 R2 | F14 R2 | F8 R3 | F2 R4 | F10 R4 | F4 R5 | F12 R5 | F7 R6 | F2 R7 | F10 R7 |
| B | F2 R1 | F10 R1 | F7 R2 | F1 R3 | F9 R3 | F3 R4 | F11 R4 | F5 R5 | F13 R5 | F8 R6 | F3 R7 | F11 R7 |
| C | F3 R1 | F11 R1 | F8 R2 | F2 R3 | F10 R3 | F4 R4 | F12 R4 | F6 R5 | F14 R5 | F9 R6 | F4 R7 | F12 R7 |
| D | F4 R1 | F12 R1 | F9 R2 | F3 R3 | F11 R3 | F5 R4 | F13 R4 | F7 R5 | F2 R6 | F10 R6 | F5 R7 | F13 R7 |
| E | F5 R1 | F13 R1 | F10 R2 | F4 R3 | F12 R3 | F6 R4 | F14 R4 | F8 R5 | F3 R6 | F11 R6 | F6 R7 | F14 R7 |
| F | F6 R1 | F14 R1 | F11 R2 | F5 R3 | F13 R3 | F7 R4 | F1 R5 | F9 R5 | F4 R6 | F12 R6 | F7 R7 | F1 R8 |
| G | F7 R1 | F3 R2 | F12 R2 | F6 R3 | F14 R3 | F8 R4 | F2 R5 | F10 R5 | F5 R6 | F14 R6 | F8 R7 | F2 R8 |
| H | F8 R1 | F4 R2 | F13 R2 | F7 R3 | F1 R4 | F9 R4 | F3 R5 | F11 R5 | F6 R6 | F1 R7 | F9 R7 | F3 R8 |

Table 20. Plate 1 map, primer combinations for both *mdh* and *trpA*

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | - | F6 R8 | - | - | - | F14 R8 |
| B | - | - | - | - | - | - |
| C | - | - | F7 R8 | - | - | - |
| D | - | - | F8 R8 | - | - | - |
| E | - | - | F9 R8 | - | - | - |
| F | - | - | F10 R8 | - | F13 R8 | - |
| G | - | - | - | F11 R8 | - | - |
| H | - | - | - | F12 R8 | - | - |

Table 21. Plate 2 map, primer combinations for both *mdh* and *trpA*

| Day | *mdh* results | *trp* results | *16s* results | Day | *mdh* results | *trp* results | *16s* results | Day | *mdh* results | *trp* results | *16s* results |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 131 | 0 | 0 | 0 | 247 | 1 | 1 | 1 |
| 8 | | | | 139 | 0 | 0 | 0 | 248 | 0 | 0 | 0 |
| 9 | 1 | 0 | 0 | 141 | 0 | 0 | 1 | 249 | 0 | 0 | 1 |
| 10 | | | | 142 | | | | 256 | 1 | 0 | 1 |
| 11 | | | | 143 | 1 | 1 | 0 | 256 | 1 | 1 | 1 |
| 12 | 0 | 0 | 1 | 180 | 0 | 0 | 0 | 257 | 0 | 0 | 0 |
| 14 | | | | 182 | | | | 258 | 1 | 1 | 0 |
| 16 | | | | 185 | 0 | 0 | 0 | 261 | 0 | 0 | 1 |
| 18 | 1 | 0 | 0 | 187 | 1 | 1 | 1 | 262 | 0 | 0 | 0 |
| 19 | | | | 188 | | | | 263 | 0 | 0 | 0 |
| 21 | | | | 191 | | | | 265 | 0 | 0 | 1 |
| 22 | | | | 192 | | | | 267 | 0 | 1 | 1 |
| 23 | 0 | 0 | 0 | 192 | | | | 269 | 1 | 0 | 1 |
| 26 | 1 | 0 | 0 | 196 | 3 | 0 | 0 | 270 | 1 | 0 | 0 |
| 31 | 1 | 0 | 0 | 197 | | | | 272 | 0 | 0 | 0 |
| 33 | | | | 198 | 0 | 0 | 0 | 275 | 0 | 0 | 1 |
| 35 | | | | 199 | | | | 279 | 0 | 0 | 0 |
| 38 | | | | 200 | 0 | 0 | 0 | 280 | 1 | 1 | 1 |
| 41 | 1 | 0 | 0 | 209 | 1 | 1 | 0 | 281 | 0 | 0 | 0 |
| 45 | 1 | 0 | 0 | 210 | 1 | 1 | 1 | 282 | 0 | 0 | 1 |
| 51 | | | | 211 | 1 | 0 | 0 | 283 | 0 | 1 | 0 |
| 55 | | | | 212 | 0 | 1 | 0 | 284 | 1 | 1 | 1 |
| 57 | 1 | 0 | 0 | 213 | 0 | 0 | 0 | 285 | 1 | 1 | 1 |
| 61 | | | | 214 | 0 | 0 | 1 | 287 | 3 | 0 | 1 |
| 62 | 0 | 0 | 1 | 215 | 1 | 1 | 1 | 288 | 0 | 0 | 1 |
| 64 | | | | 216 | 0 | 0 | 0 | 289 | 0 | 0 | 0 |
| 66 | | | | 217 | 1 | 0 | 0 | 328 | 0 | 1 | 1 |
| 67 | 0 | 0 | 1 | 218 | 1 | 0 | 1 | 329 | 1 | 0 | 1 |
| 68 | 1 | 1 | 1 | 222 | 0 | 0 | 1 | 330 | 1 | 0 | 0 |
| 69 | 0 | 0 | 1 | 223 | 1 | 1 | 1 | 331 | 0 | 0 | 1 |
| 71 | | | | 225 | 1 | 0 | 0 | 334 | 1 | 0 | 1 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 73 | | | | 226 | 0 | 1 | 1 | 337 | 1 | 0 | 1 |
| 74 | 1 | 1 | 0 | 227 | 0 | 1 | 1 | 345 | 0 | 0 | 0 |
| 75 | | | | 229 | 0 | 0 | 1 | 349 | 1 | 3 | 1 |
| 76 | | | | 230 | 1 | 1 | 0 | 350 | 0 | 0 | 0 |
| 77 | | | | 231 | 3 | 1 | 1 | 351 | 1 | 1 | 1 |
| 79 | 0 | 1 | 0 | 236 | 0 | 0 | 1 | 352 | 1 | 0 | 1 |
| 96 | 1 | 1 | 1 | 237 | 1 | 0 | 0 | 357 | 1 | 1 | 1 |
| 105 | 1 | 1 | 1 | 238 | 0 | 0 | 1 | 359 | 1 | 0 | 0 |
| 112 | 1 | 1 | 1 | 239 | 0 | 1 | 1 | 361 | 0 | 0 | 0 |
| 113 | | | | 241 | 0 | 0 | 1 | 362 | 1 | 0 | 1 |
| 122 | 0 | 0 | 1 | 242 | 0 | 1 | 1 | 363 | 0 | 0 | 1 |
| 126 | 1 | 0 | 0 | 244 | 1 | 0 | 1 | 365 | 0 | 0 | 0 |
| 128 | | | | 245 | 0 | 0 | 0 | | | | |

Table 22. Results of initial amplification attempts for all sample. 1 indicates successful amplification. 0 indicates unsuccessful amplification or ambiguous results. Blank indicates amplification was not attempted.

| | *mdh*1 | *mdh*2 | *mdh*5 | *mdh*8 | *mdh*35 | *mdh*36 | *mdh*60 | *mdh*85 | *mdh*96 | *mdh*122 | *mdh*130 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *mdh*1 | 0,000 | 0,009 | 0,013 | 0,009 | 0,017 | 0,017 | 0,023 | 0,015 | 0,017 | 0,013 | 0,017 |
| *mdh*2 | 0,009 | 0,000 | 0,007 | 0,004 | 0,011 | 0,011 | 0,013 | 0,009 | 0,007 | 0,004 | 0,011 |
| *mdh*5 | 0,013 | 0,007 | 0,000 | 0,011 | 0,019 | 0,019 | 0,021 | 0,002 | 0,015 | 0,011 | 0,019 |
| *mdh*8 | 0,009 | 0,004 | 0,011 | 0,000 | 0,015 | 0,015 | 0,017 | 0,013 | 0,011 | 0,007 | 0,015 |
| *mdh*35 | 0,017 | 0,011 | 0,019 | 0,015 | 0,000 | 0,004 | 0,021 | 0,020 | 0,015 | 0,011 | 0,015 |
| *mdh*36 | 0,017 | 0,011 | 0,019 | 0,015 | 0,004 | 0,000 | 0,021 | 0,020 | 0,015 | 0,011 | 0,015 |
| *mdh*60 | 0,023 | 0,013 | 0,021 | 0,017 | 0,021 | 0,021 | 0,000 | 0,023 | 0,017 | 0,017 | 0,021 |
| *mdh*85 | 0,015 | 0,009 | 0,002 | 0,013 | 0,020 | 0,020 | 0,023 | 0,000 | 0,017 | 0,013 | 0,021 |
| *mdh*96 | 0,017 | 0,007 | 0,015 | 0,011 | 0,015 | 0,015 | 0,017 | 0,017 | 0,000 | 0,011 | 0,011 |
| *mdh*122 | 0,013 | 0,004 | 0,011 | 0,007 | 0,011 | 0,011 | 0,017 | 0,013 | 0,011 | 0,000 | 0,015 |
| *mdh*130 | 0,017 | 0,011 | 0,019 | 0,015 | 0,015 | 0,015 | 0,021 | 0,021 | 0,011 | 0,015 | 0,000 |

Table 23. Pairwise-distance matrix for *mdh* alleles computed using the maximum composite likelihood model in MEGA7.

| | *trpA*_1 | *trpA*_2 | *trpA*_8 | *trpA*_10 | *trpA*_12 | *trpA*_19 | *trpA*_36 | *trpA*_139 |
|---|---|---|---|---|---|---|---|---|
| *trpA*_1 | 0,000 | 0,062 | 0,009 | 0,064 | 0,062 | 0,067 | 0,011 | 0,031 |
| *trpA*_2 | 0,062 | 0,000 | 0,064 | 0,004 | 0,043 | 0,026 | 0,062 | 0,048 |
| *trpA*_8 | 0,009 | 0,064 | 0,000 | 0,066 | 0,062 | 0,069 | 0,009 | 0,033 |
| *trpA*_10 | 0,064 | 0,004 | 0,066 | 0,000 | 0,043 | 0,025 | 0,064 | 0,050 |
| *trpA*_12 | 0,062 | 0,043 | 0,062 | 0,043 | 0,000 | 0,035 | 0,062 | 0,060 |
| *trpA*_19 | 0,067 | 0,026 | 0,069 | 0,025 | 0,035 | 0,000 | 0,066 | 0,054 |
| *trpA*_36 | 0,011 | 0,062 | 0,009 | 0,064 | 0,062 | 0,066 | 0,000 | 0,035 |
| *trpA*_139 | 0,031 | 0,048 | 0,033 | 0,050 | 0,060 | 0,054 | 0,035 | 0,000 |

Table 24. Pairwise-distance matrix for *trpA* alleles computed using the maximum composite likelihood model in MEGA7.

| Primers | F9R7 | F7R8 | F7R7 | F8R7 | F1R8 | F11R7 | F2R8 | F13R8 | F10R7 | F6R7 | F12R8 | F14R8 | F6R8 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample number | 85 | 109 | 83 | 84 | 91 | 87 | 92 | 128 | 86 | 82 | 122 | 131 | 99 |
| Day | 9 | 18 | 26 | 31 | 41 | 45 | 57 | 68 | 74 | 79 | 96 | 105 | 112 |
| *mdh* 1 | 0 | 12 | 0 | 0 | 0 | 0 | 86 | 17 | 0 | 0 | 18 | 81 | 85 |
| *mdh* 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 28 | 0 | 0 | 0 |
| *mdh* 35 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 60 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 8 | 4 | 0 | 18 | 0 | 0 | 21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 85 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 96 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 122 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 130 | 20 | 0 | 48 | 39 | 6 | 10 | 0 | 0 | 10 | 0 | 0 | 0 | 0 |
| Junked reads | 11 | 14 | 22 | 6 | 2 | 8 | 22 | 7 | 5 | 22 | 15 | 37 | 35 |
| Total | 35 | 26 | 88 | 45 | 8 | 39 | 108 | 24 | 15 | 50 | 33 | 118 | 120 |

| Primers | F14R7 | F13R2 | F12R7 | F14R4 | F7R1 | F11R2 | F4R1 | F9R1 | F4R6 | F5R4 | F11R1 | F10R5 | F14R1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample number | 90 | 22 | 88 | 50 | 5 | 20 | 2 | 7 | 67 | 42 | 9 | 60 | 12 |
| Day | 143 | 187 | 196 | 209 | 214 | 215 | 218 | 223 | 230 | 237 | 239 | 244 | 247 |
| *mdh* 1 | 206 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 2 | 0 | 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 23 | 5 | 8 | 0 |
| *mdh* 35 | 0 | 0 | 9 | 0 | 0 | 0 | 0 | 7 | 4 | 8 | 0 | 0 | 99 |
| *mdh* 36 | 0 | 0 | 10 | 785 | 171 | 55 | 24 | 20 | 9 | 0 | 0 | 0 | 0 |
| *mdh* 5 | 0 | 25 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 0 | 17 | 0 | 0 |
| *mdh* 60 | 0 | 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 15 | 0 | 0 |
| *mdh* 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 7 | 0 |
| *mdh* 85 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 96 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 122 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 130 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Junked reads | 49 | 64 | 10 | 290 | 81 | 55 | 39 | 32 | 15 | 49 | 232 | 19 | 93 |
| Total | 255 | 125 | 29 | 1075 | 252 | 118 | 63 | 59 | 34 | 80 | 269 | 34 | 192 |

| Primers | F14R3 | F4R4 | F6R3 | F12R4 | F5R1 | F3R2 | F14R2 | F10R2 | F6R2 | F9R5 | F7R2 | F9R2 | F10R1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample number | 37 | 41 | 29 | 48 | 3 | 13 | 23 | 19 | 15 | 59 | 16 | 18 | 8 |
| Day | 256 | 258 | 267 | 270 | 280 | 284 | 287 | 329 | 334 | 337 | 349 | 351 | 357 |
| *mdh* 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 2 | 0 | 0 | 0 | 0 | 0 | 0 | 16 | 12 | 0 | 0 | 4 | 0 | 0 |
| *mdh* 35 | 123 | 306 | 236 | 535 | 108 | 127 | 0 | 0 | 6 | 0 | 0 | 0 | 0 |
| *mdh* 36 | 11 | 0 | 0 | 6 | 0 | 20 | 0 | 0 | 51 | 0 | 0 | 0 | 0 |
| *mdh* 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 0 |
| *mdh* 60 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 47 | 111 | 18 |
| *mdh* 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 31 | 0 | 0 | 0 |
| *mdh* 85 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 22 | 0 | 4 | 0 | 0 | 0 |
| *mdh* 96 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 122 | 0 | 0 | 0 | 0 | 0 | 0 | 12 | 8 | 0 | 0 | 0 | 0 | 0 |
| *mdh* 130 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Junked reads | 42 | 87 | 55 | 171 | 72 | 60 | 23 | 23 | 39 | 19 | 122 | 30 | 59 |
| Total | 176 | 393 | 291 | 712 | 180 | 207 | 51 | 74 | 96 | 54 | 178 | 141 | 77 |

| Primers | F4R2 | F4R8 | F5R8 |
|---|---|---|---|
| Sample number | 14 | Cust.1 | Cust.2 |
| Day | 362 | | |
| *mdh* 1 | 0 | 0 | 0 |
| *mdh* 2 | 0 | 0 | 0 |
| *mdh* 35 | 0 | 0 | 0 |
| *mdh* 36 | 0 | 0 | 0 |
| *mdh* 5 | 0 | 0 | 0 |
| *mdh* 60 | 78 | 0 | 0 |
| *mdh* 8 | 0 | 112 | 24 |
| *mdh* 85 | 0 | 0 | 0 |
| *mdh* 96 | 0 | 0 | 0 |
| *mdh* 122 | 0 | 0 | 0 |
| *mdh* 130 | 0 | 0 | 130 |
| Junked reads | 36 | 28 | 175 |
| Total | 114 | 140 | 329 |

Table 25. Number of reads of each *mdh* sequence variant identified for each sample.

| Primers | F9R7 | F8R7 | F2R8 | F13R8 | F10R7 | F6R7 | F12R8 | F14R8 | F6R8 | F14R7 | F13R2 | F12R7 | F14R4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample number | 85 | 84 | 92 | 128 | 86 | 82 | 122 | 131 | 99 | 90 | 22 | 88 | 50 |
| Day | 9 | 31 | 57 | 68 | 74 | 79 | 96 | 105 | 112 | 143 | 187 | 196 | 209 |
| *trpA* 1 | 79 | 79 | 103 | 156 | 0 | 0 | 36 | 386 | 191 | 75 | 0 | 0 | 0 |
| *trpA* 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 66 | 2 |
| *trpA* 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 42 | 0 | 0 |
| *trpA* 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 12 | 89 | 0 |
| *trpA* 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *trpA* 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *trpA* 36 | 0 | 0 | 0 | 0 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *trpA* 139 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Junked reads | 28 | 28 | 36 | 43 | 12 | 6 | 11 | 103 | 55 | 38 | 34 | 63 | 1 |
| Total | 107 | 107 | 139 | 199 | 16 | 8 | 47 | 489 | 246 | 113 | 88 | 218 | 3 |

| Primers | F11R2 | F4R1 | F9R1 | F11R1 | F10R5 | F14R1 | F14R3 | F4R4 | F6R3 | F5R1 | F3R2 | F14R2 | F6R1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample number | 20 | 2 | 7 | 9 | 60 | 12 | 37 | 41 | 29 | 3 | 13 | 23 | 4 |
| Day | 215 | 218 | 223 | 239 | 244 | 247 | 256 | 258 | 267 | 280 | 284 | 287 | 328 |
| *trpA* 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9 |
| *trpA* 2 | 12 | 42 | 15 | 0 | 76 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *trpA* 8 | 0 | 0 | 0 | 20 | 100 | 4 | 0 | 0 | 0 | 0 | 0 | 27 | 0 |
| *trpA* 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *trpA* 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *trpA* 19 | 0 | 0 | 0 | 0 | 4 | 223 | 60 | 3 | 89 | 37 | 44 | 16 | 0 |
| *trpA* 36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *trpA* 139 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Junked reads | 8 | 67 | 15 | 16 | 102 | 125 | 37 | 2 | 28 | 9 | 26 | 35 | 12 |
| Total | 20 | 109 | 30 | 36 | 282 | 352 | 97 | 5 | 117 | 46 | 70 | 78 | 21 |

| Primers | F10R2 | F6R2 | F9R5 | F7R2 | F9R2 | F10R1 | F4R8 | F5R8 |
|---|---|---|---|---|---|---|---|---|
| Sample number | 19 | 15 | 59 | 16 | 18 | 8 | Cust.1 | Cust.2 |
| Day | 329 | 334 | 337 | 349 | 351 | 357 | | |
| *trpA* 1 | 0 | 75 | 0 | 0 | 0 | 0 | 0 | 0 |
| *trpA* 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *trpA* 8 | 6 | 0 | 0 | 0 | 0 | 0 | 165 | 22 |
| *trpA* 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *trpA* 12 | 13 | 12 | 19 | 0 | 0 | 0 | 0 | 0 |
| *trpA* 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *trpA* 36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 96 |
| *trpA* 139 | 0 | 0 | 0 | 15 | 71 | 4 | 0 | 0 |
| Junked reads | 9 | 43 | 9 | 2 | 23 | 9 | 57 | 255 |
| Total | 28 | 130 | 28 | 17 | 94 | 13 | 222 | 373 |

Table 26. Number of reads of each *trpA* sequence variant identified for each sample.

## Appendix 3: Visualization of gel electrophoresis of PCR products.

Figure 16. Prototype testing scheme with *mdh* F1-R1

Figure 17. Prototype testing scheme with *mdh* F2-R2, F3-R3, F4-R4, and F5-R5. The negative control or F4-R4 has swapped places with one of the positives due to a pipetting error.

Figure 18. Large-scale  testing scheme with *mdh* R1, R2, and R3 combinations.

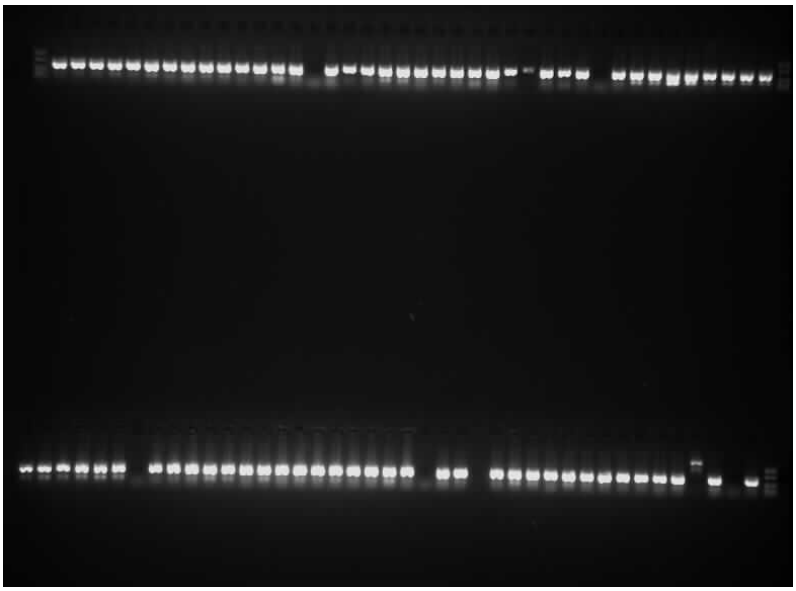


Figure 19. Large-scale  testing scheme with *mdh* R2, R4, R5, R6 and R7 combinations.

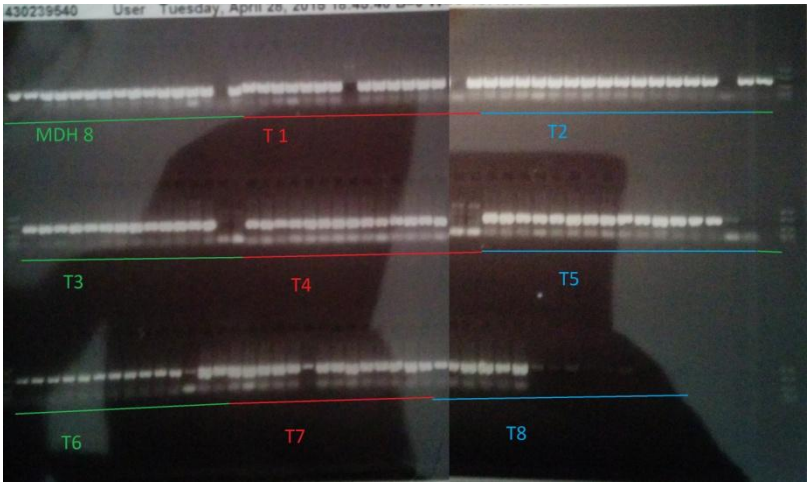



Figure 20. Large-scale  testing scheme with *mdh* R8, and *trpA* R1, R2, R3, R4, R5, R6, R7, and R8 combinations.

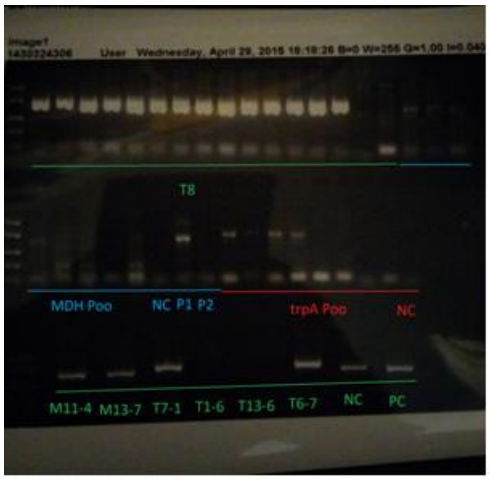



Figure 21. Large-scale testing scheme with *trpA* R8 combinations, fecal DNA controls, and redos from previous runs.

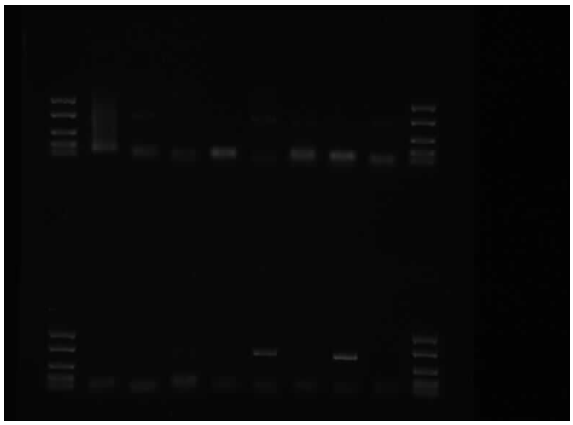


Figure 22. Trial run using randomly picked primers and samples

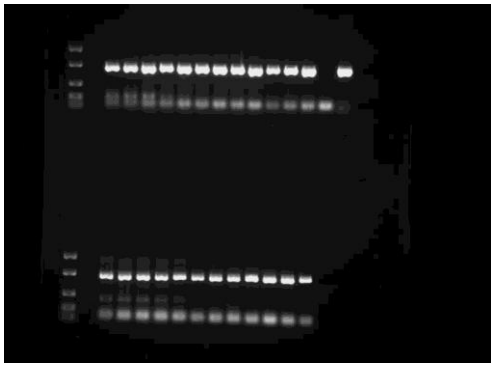


Figure 23. Gradient PCR to determine optimal annealing temperature.

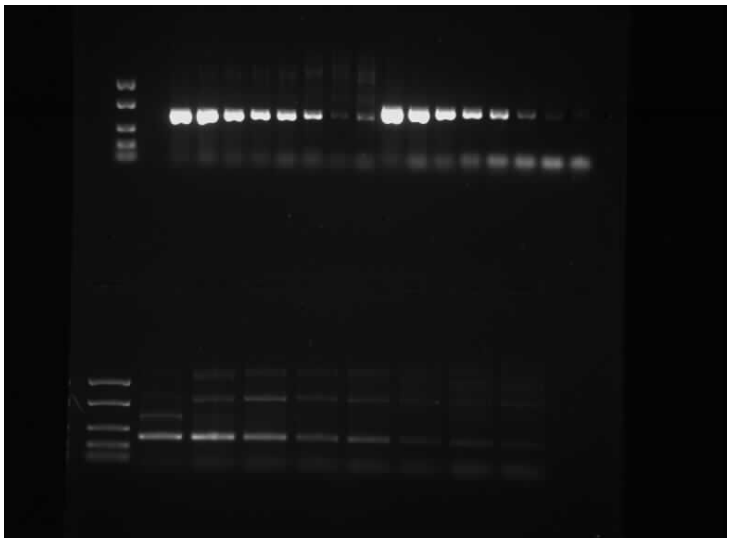


Figure 24. PCR of template dilution series to determine lower detection limit for primers.

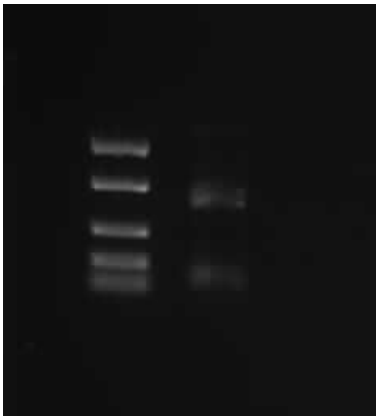


Figure 25. Electrophoresis of purified sequencing pool.

## Appendix 4: Sequencing report


NORWEGIAN SEQUENCING CENTRE

### Sequencing results for sample Infant E.coli pool (NSC sample ID: PB_0267)

#### 1. Library preparation

Library was prepared using Pacific Biosciences 1 kb library preparation protocol. Size selection of the final library was performed using Ampure Beads.

#### 2. Sequencing

The library was sequenced on Pacific Biosciences RS II instrument using P6-C4 chemistry, movie time 360 minutes, one SMRT cell was used for sequencing.

**Results:**

| | |
|---|---|
| Number of reads: | 46 842 |
| Average polymerase read length: | 22 703 bp |
| Total number of polymerase bases: | 1 063.4 Mb |
| Average read of insert length: | 1098 bp |

#### 3. Filtering using Reads of Insert pipeline on SMRT Portal

3.1. Reads were filtered using RS_subreads.1 pipeline on SMRT Portal (SMRT Analysis version smrtanalysis_2.3.0.140936.p2.144836) with default settigs (minimum accuracy 0.90, minimum 1 pass)

**Job Metric:**

| | |
|---|---|
| Reads of Insert | 32 048 |
| Read Bases of Insert | 28.77 Mb |
| Mean Read Length of Insert | 897 bp |
| Read Accuracy of Insert | 99.21% |
| Mean Number of Passes | 31.41 |

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