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The Effect of Freezing on Faecal Microbiota Transplants' Microbe Composition

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Faecal Microbiota Transplantation is a treatment in which the faeces of a healthy person are transferred into a patient to restore the balance of their gut microbe composition. As it has become a more common treatment in Western medicine during the last decade, the need for faecal microbiota transplant providers such as Turku Clinical Microbiome Bank has increased. To provide safe and effective faecal microbiota transplantations, the effects of long-term frozen storage, transplant processing and individual donors on the microbe composition of the transplant need to be analysed. This study aims to analyse the significance of these effects on faecal microbiota transplants in order to validate and improve the protocols used in Turku Clinical Microbiome Bank. Additionally, the effects of 16S V3V4 rRNA sequencing, shotgun sequencing and different analysing methods on results and conclusions were analysed.

The main results of this research were that frozen storage for up to three years and transplant processing do not statistically significantly affect the microbe compositions of the transplants. However, differences between donors and genders (p-values <0.0001) were observed. The sequencing and analysing methods also differed from each other but the differences did not affect conclusions.

As no statistically significant differences due to frozen storage were found, further studies should be performed to conclude how long the transplants can be stored without affecting their microbe composition. Additionally, the differences observed between genders should be confirmed with a larger sample size.

Keywords: Faecal Microbiota Transplantation, frozen storage, gut microbiota composition.

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1 Introduction

Faecal microbiota transplants, otherwise known as stool transplants, are essentially gut microbe solutions made from the faeces of a healthy donor. These gut microbe solutions are made to help restore the gut microbe composition of a person suffering from gut dysbiosis, which can manifest itself, for example, as diarrhoea. Faecal microbiota transplantation (FMT) has been used for the treatment of diarrhoea since 4th century China (Wang et al., 2019). However, in Western medicine, it has become more common only during the last decade after its effectiveness in treating recurrent *Clostridioides difficile* infection (CDI) was proven (van Nood et al., 2013).

CDI is the second most common cause of diarrhoea in Finland, affecting 4000-5000 people yearly (Anttila, 2022). It is usually triggered by antibiotic treatment of another condition and is therefore difficult to prevent. Its first-line treatments are antibiotics such as metronidazole, vancomycin or fidaxomicin, which are used to remove the harmful *Clostridioides difficile* bacteria from the gut. However, approximately every sixth person with CDI has a relapse after the antibiotic treatment (Anttila, 2022). These relapses can be treated with FMT which has an 81-92% overall cure rate depending on whether the patients are treated only once or repeatedly in case of failed treatments (Porcari et al., 2023a). The effectiveness of FMT has also been shown to increase when the amount of stool given is 50-212g (Ianiro et al., 2018). The benefit of FMT compared to antibiotics is that it does not damage the beneficial gut microbes but rather supports and increases them so that they can regain control in the gut.

The effectiveness of FMT in treating other gastrointestinal diseases such as inflammatory bowel disease (IBD) (Kim and Gluck, 2019) and irritable bowel syndrome (IBS) (El-Salhy et al., 2021a, 2022) has also been studied, but so far, the evidence of the benefits of FMT for these conditions is insufficient. In general, both diseases have lower cure rates with FMT than recurrent CDI, although not all of the studies are able to find statistically significant differences between FMT and placebo treatment for these conditions. Apart from these two gastrointestinal diseases, there is also an increasing amount of research ascertaining whether FMT is effective in treating neurological diseases such as Parkinson's disease (Xue et al., 2020), mood disorders (Chinna Meyyappan et al., 2020), autism spectrum disorder (Aroniadis and Brandt, 2013) and many others (Hamamah et al., 2022). Even though there are promising results for some of these conditions, more research is still needed to ascertain the complete risks and benefits of FMT for each one of them. Furthermore, the mechanisms behind efficacious FMT should be studied more, so that all patients can receive safe and effective treatment.

In the future, FMT could also be used as personalised medicine. For example, donors and patients could be paired together based on the compatibility of their microbiotas. This could help increase the specific beneficial microbes missing from the patient's microbiota as well as increase the amount and persistence of microbial engraftment. Another possible development with these treatments could be providing stool transplants to oneself when the normal gut microbiota composition has been disturbed. This disturbance could be caused, for example, by antimicrobial treatment, time spent in a foreign country or a sudden occurrence of a disease that could be treated with FMT, like CDI. The difficulty in this treatment being personalized is that the donor/patient should be healthy at the time of donation after which they suddenly or perhaps expectedly, in case of travels, fall ill and require treatment. The longer the stool transplants can be stored, the more feasible these kinds of treatments and preventative measures become. However, longer storage times also cause an increased need for larger storage facilities which in turn could increase the costs of this treatment. In the end, using the faeces of a donor is probably easier than trying to anticipate the need for FMT.

On the other hand, there has also been discussion and research on multi-donor FMT. Multi-donor FMT has more than one donor's faeces in the stool transplant. This is thought to increase the number and diversity of microbes in the transplant, consequently also increasing the probability of the treatment having a beneficial effect. The disadvantage of using several donors is that even though it can increase the microbe count and diversity of the transplant, it also increases the risk of transmitting a pathogen to the patient. However, this risk should be relatively low if all donors have been screened well. Additionally, not all gut microbiotas are compatible with each other meaning that combining several well-balanced microbiotas might not always result in an improved stool transplant composition. However, so far there are no studies comparing single- and multi-donor transplants to each other (Porcari et al., 2023b), and therefore, it remains unknown whether multi-donor transplants are superior to transplants made from one donor.

1.1 Delivery methods

The stool transplants can be given to patients through various upper and lower gastrointestinal delivery methods. The most common ones include colonoscopy, retention enema, jejunal infusion, nasoduodenal tube and capsules. As FMT is mostly used to treat gastrointestinal diseases, which are commonly associated with problems with the integrity of the gut, such as leaky tight junctions, permeable gut epithelium and low mucus secretion, the delivery methods with tubes (Gulati et al., 2020) can be risky. The lower gastrointestinal delivery procedures can cause, for example, further

irritation of the gut walls, bleeding or even perforation of the gut (Kim and Gluck, 2019). Upper gastrointestinal delivery methods like nasoduodenal delivery, on the other hand, can have respiratory tract and stomach-related problems such as aspiration and vomiting (Wang et al., 2016; Kim and Gluck, 2019). In general, upper gastrointestinal delivery methods have more general and minor adverse effects than lower gastrointestinal delivery methods but the roles are reversed when it comes to serious adverse effects (Wang et al., 2016).

The newest delivery route option is orally by capsule. The capsules are a promising development as they do not cause mechanical harm to the gut walls, they are patient-friendly as the treatment can be given without invasive procedures and they are usually cheaper to produce (Kao et al., 2017). However, since the capsules contain lower amounts of faeces than regular stool transplants, there is often a need to ingest large quantities, such as 40 capsules (Kao et al., 2017), within a few days to match the overall amount needed for successful treatment. The adverse effects associated with the capsules are minor including nausea, vomiting and abdominal pains (Kao et al., 2017; Greenberg et al., 2018) which can also occur with the other delivery methods.

The delivery methods also differ somewhat in efficacy. The upper gastrointestinal delivery methods are usually the least effective while lower gastrointestinal delivery methods are the most effective (Ianiro et al., 2018). Colonoscopy is generally the most common and effective delivery method, but it is not statistically significantly different from other lower gastrointestinal delivery methods or capsules (Kao et al., 2017; Ianiro et al., 2018). Therefore, any one of these methods should provide the same level of effectiveness to the patient. Consequently, it is up to the clinician to decide which method is used for each patient as none of the delivery methods is clearly better than the others.

1.2 Mode of action

As FMT has become a more common treatment option, the demand for it has also increased. This has posed a lot of difficulties for FMT providers, such as stool banks, as suitable donors are difficult to come by and reliable information about the best stool transplantation practices has been scarce. Since FMT was proven as an effective treatment for CDI, a lot of research has been performed to find out how the faeces should be processed into stool transplants without affecting the original microbe composition of the faeces or the effectiveness of the transplant. In addition to this, some research has been performed to determine how different storage methods, temperatures and durations affect the quality of the transplants. However, it is still unclear what exactly the mechanism or mechanisms through which the FMT improves the disease states of patients are and whether these mechanisms differ significantly between diseases.

The main curative effects of FMT are considered to be caused by microbial engraftment to the gut. In CDI patients the gut microbiota is commonly deficient in *Bacteroidetes* and *Firmicutes* phyla while *Proteobacteria* are overly abundant. Following FMT, the abundances of *Bacteroidetes* and *Firmicutes* as well as overall species richness all increase (Khoruts et al., 2021) which helps to correct the bacterial imbalance associated with CDI. Other factors that could be meaningful for the effectiveness of FMT in treating CDI are a decreased ratio of primary and secondary bile acids, altered immune responses (Khoruts and Sadowsky, 2016) and an increase in short-chain fatty acids (El-Salhy et al., 2021b) all of which are byproducts of the successful microbial engraftment. When the FMT is successful, the engraftment can persist for months or even years (Khoruts and Sadowsky, 2016) providing long-term relief and protection against CDI recurrence.

1.3 Donor selection

A lot of research has been conducted on the best way to collect and process stool transplants since the first clinical randomized trial in 2013 (van Nood et al., 2013). Based on the results of these studies, a consensus on stool bank protocols was published in 2019 (Cammarota et al., 2019). According to it, faecal microbiota donors should be selected based on extensive screening and tests, so that any disease transmission through the transplant can be prevented.

The screening methods include checking the medical history and travel records of the donor applicant as well as a discussion with the applicant to ascertain risks of infection or disturbed gut microbiota. The physiological tests include blood tests which are used to check for hepatitis, HIV, *Treponema pallidum* and nematodes infections, and to see whether the blood cell count, creatinine and aminotransferase values are normal. The faeces of the donor applicant should also be tested to check that it does not include any common pathogens such as *Clostridioides difficile*, *Salmonella*, antibiotic-resistant bacteria, Norovirus, parasites or *Giardia lamblia*. (Cammarota et al., 2019)

1.4 FMT processing

Based on previous studies, it has been determined that at least 25 g of faeces should be used for lower gastrointestinal delivery and 12.5 g for upper gastrointestinal delivery to ensure the effectiveness of the treatment. If the transplants are frozen for later use, a cryoprotectant such as glycerol (10%) should be added to the faeces before freezing at -80°C to ensure longer viability of the gut microbes. The entire processing should be completed within six hours of the donation to preserve the original microbe composition of the faeces. Additionally, the FMT should be

performed within six hours after thawing the frozen transplant at room temperature or at a 37°C water bath. (Cammarota et al., 2019)

Several studies prove that fresh and frozen stool transplants are equal in efficacy (Quraishi et al., 2017; Kao et al., 2017; Ianiro et al., 2018; Kim and Gluck, 2019). This means that stool banks can collect and store faeces in large quantities and subsequently use them when needed instead of having to call the donor to donate every time a transplant is needed. However, most of the published studies focus on differences between fresh and frozen transplants only up to six months of frozen storage (Costello et al., 2015a), which has not been enough time to show a difference in the efficacies of the two treatment types. This is problematic as the current recommendation for the frozen storage duration of stool transplants is up to two years (Cammarota et al., 2019).

There are some studies that have evaluated the differences between the efficacy of fresh and frozen stool transplants with over six months of frozen storage (Elliott et al., 2016; Jiang et al., 2017), but only some of them reach a point at which the efficacy of the frozen stool transplants starts to decrease. Furthermore, some studies including the longest study with two years of frozen storage (Elliott et al., 2016) measure the change in efficacy by only observing patient outcomes without any quantitation of the microbe composition changes of the transplants. Consequently, a limited amount of data is available about the changes in the microbe composition of the transplants over long periods of frozen storage. However, some studies do have results from the gut microbiota composition of the patient before and after treatment (Halkjær et al., 2018). These studies are important, as they provide some information about which bacteria are able to survive the freezing and engraftment, and also which bacteria might be important for the effectiveness of the FMT.

1.5 Aims and hypothesis

This research aims to determine whether and at which point the microbe composition of the faeces changes statistically significantly over three years of frozen storage at -80°C. The samples are collected from three different steps of the stool transplant processing to check whether the processing has a statistically significant effect on the microbe composition. Additionally, the differences between donors and donations are studied to reach a better understanding of the factors that can affect the microbe composition of the faecal microbiota transplant. Furthermore, 16S rRNA and shotgun sequencing and several bioinformatics analysis tools are compared to determine reliable ways to analyse gut microbiota data.

Based on the previous evidence, it is expected that the stool transplant processing does not affect the gut microbes statistically significantly (Costello et al., 2015b; Satokari et al., 2015) while freezing at -80°C is expected to have an effect at some point during the three years. The freezing should reduce the number of microbes in the samples due to the increased stress caused by freezing and thawing. It is also expected that different analysing methods yield differing results which might sometimes lead to differing conclusions.

There is still very little information available on how freezing at -80°C affects the gut microbes over a long period of time such as three years. The use of frozen stool transplants is currently restricted to two years of frozen storage even though there is limited information available to support even the two-year mark (Cammarota et al., 2019; Elliott et al., 2016). It is important to increase the understanding of the effects freezing has on gut microbes so that reliable and evidence-based FMT treatments can be provided to patients. This research strives to show how well gut microbes fare while being frozen from less than a year up to three years. Additionally, as each stool bank has its own protocols, it is also important to be able to validate and possibly improve the protocols used in the Turku Clinical Microbiome Bank whose samples were used to perform this study. As this stool bank is one of the few FMT providers in Finland and its processes have not yet been validated in this manner, it is all the more important to gain overall information about the quality of this FMT process.

1.6 Sequencing methods

1.6.1 16S rRNA sequencing

16S ribosomal RNA (rRNA) sequencing is a common technique that has been used for microbial and archaeal analyses since 1977 (Kim and Chun, 2014). As all organisms need protein synthesis to survive, the ribosomes are vital. Consequently, the rRNA genes exist in all species and can therefore be used for genus and species-level identification from DNA sequences. However, to perform taxonomical classification accurately based on one gene, it is not only important that all species express the gene but also that it differs between most species. This is why the small subunit of the rRNA gene is often used in taxonomical classification as it contains several hypervariable regions which differ between species. (López-Aladid et al., 2023)

In total, there are nine hypervariable regions in the small subunit of the rRNA gene that can be used to assign taxonomies. Depending on the type of microbes being classified, different combinations of these regions can be used, such as V1-V3 and V3-V4. (Johnson et al., 2019; López-Aladid et al.,

2023) The whole 16S rRNA gene is not typically sequenced because the costs are higher and not many sequencing equipment are able to sequence long enough sequences. However, the costs have reduced and newer long-read sequencing techniques such as PacBio and Oxford Nanopore have entered the market recently and therefore it could be possible to start sequencing the whole gene more commonly. This would help to increase the reliability of the taxonomical classifications of 16S sequencing. (Johnson et al., 2019)

16S rRNA sequencing is not a perfect way to assign taxonomies to sequences as it often reaches only genus-specific microbial identification. Some sequences can be classified up to the species level, but in general, the genus level is thought to be more reliable (Johnson et al., 2019). The final taxonomical classification of the sequences can also be affected by mistakes during PCR amplification and sequencing as the regions being sequenced are quite short (Abellan-Schneyder et al., 2021). The abundance of these mistakes has however decreased as the sequencing techniques have improved. Nevertheless, in general, the longer the sequenced regions are the more reliable the taxonomical classifications are as well.

1.6.2 Shotgun sequencing

Shotgun sequencing is a technique in which the whole genomes of the microbes in a sample are randomly fragmented, multiplied with PCR in some protocols, purified and sequenced. After sequencing, the reads can be assembled back together into a longer sequence similar to the whole genome by finding overlapping regions. (Raghavendra and Pullaiah, 2018) However, it is common that the genomes cannot be reconstructed perfectly as some pieces of the sequence might not be available which creates holes in the complete sequence. There can also be some regions like long repeating nucleotide cycles or repeating genes which are difficult to interpret correctly and therefore hinder the reconstruction of the genome (Quince et al., 2017).

The benefit of shotgun sequencing is that the amount of data is high and diverse. This makes it possible to conduct more in-depth analyses, like antibiotic resistance gene and functional profile analysis. It usually also reaches species-level taxonomical classifications which in turn make it possible to reach in-depth conclusions based on the data. However, as the sequences are random there might be some that cannot be assigned to any specific taxonomy due to their universal nature or lack of matching reference sequences.

1.7 Reference databases

Reference databases are used to taxonomically classify sequences. The database options differ in size, sequence types and quality of data. In this study, Greengenes (DeSantis et al., 2006) and SILVA (Pruesse et al., 2007) databases are used with the 16S V3V4 rRNA sequences while the Unified Human Gastrointestinal Genome (UHGG) database (Almeida et al., 2021) is used with shotgun sequences.

The reference databases provide a more reliable source of sequences, as not all publicly available sequences are correctly annotated or sequenced. In sequencing, especially with PCR multiplied sequences, many types of errors can occur (Pfeiffer et al., 2018). The sequences can break into small pieces, there can be sequencing errors and two or more sequences can be copied as one creating chimaeras. As these sequences with errors do not exist in the original samples, they also do not represent them correctly. Consequently, it is important that reliable reference databases exist and are used in data analysis so that sequences are not misclassified and sequences with major errors are not included in the analysis.

1.7.1 Greengenes

Greengenes is one of the commonly used 16S rRNA reference databases. It was created in 2006 to help provide reliable and accessible 16S rRNA sequence data. It consists of annotated, chimaera-checked, full-length 16S rRNA gene sequences of both bacterial and archaeal descent (DeSantis et al., 2006) which can be used to assign taxonomy to small subunit rRNA gene sequences (Glöckner et al., 2017). Additionally, it has been recently updated to a more extensive version called Greengenes2 (McDonald et al., 2023) which is not unfortunately included in the database comparisons of this study.

1.7.2 SILVA

The SILVA database is a more comprehensive 16S rRNA reference database than Greengenes as it also includes eukaryotic and large subunit rRNA gene sequences in addition to the bacterial and archaeal small subunit sequences (Quast et al., 2013). It was first released in 2007 (Glöckner et al., 2017), so both databases have existed for almost equally long. The sequences in the database have been taxonomically classified by several sites, their quality has been checked and the latest valid nomenclature has been assigned to them. The database is also updated twice a year without removing access to the previous versions of the database. This is useful, as it allows accurate data

analysis comparisons to be conducted to old studies even after the database has been updated. (Quast et al., 2013)

1.7.3 UHGG

The UHGG database is comprised of the whole genome sequences of human gut microbes (Almeida et al., 2021). Therefore, it has a lot more comprehensive collection of gut microbiota sequences than either of the 16S rRNA sequence databases which only consist of varying versions of one gene. The UHGG database is used to taxonomically classify whole genome sequenced data, as the probabilities of finding a match for an arbitrary microbial sequence are higher the more comprehensive the database is.

1.8 Taxonomical classification

In 16S rRNA data analysis, the sequences can be taxonomically classified with two methods. The sequences can be mapped to the reference database with a 95-99% similarity threshold, with 97% being the most commonly used similarity percentage, and grouped together based on these similarities under operational taxonomic units (OTU) (Tapolczai et al., 2019). The OTUs will then be used for further analyses. As the OTUs do not contain only exact sequence matches, some sequences in them may be taxonomically misclassified. However, having a lower similarity threshold can also reduce the effects of sequencing errors on results.

The other method is to assign taxonomies to the sequences only when they have a 100% match in the reference database. This method is called amplicon sequence variant (ASV) assigning (Callahan et al., 2017) and it results in only exact matches being used in the analysis. Therefore, the classifications should be more accurate and reliable than OTUs. However, the sequences might still be assigned to the wrong taxonomy or not be assigned taxonomy at all due to sequencing errors or lacking reference sequences, consequently resulting in a loss of information.

In shotgun sequencing, the sequences are not from any specific region like in 16S rRNA sequencing. Therefore, the sequences are taxonomically classified by mapping them to a whole genome reference database such as UHGG (Almeida et al., 2021) before or after assembling the original sequences into longer ones. The taxonomical classifications consist of all the sequences matching to them which is why the classifications do not include specific sequence information. This lack of a specific sequence per classification is unfortunate as phylogenetic analyses cannot be performed without them.

1.9 Statistical analysis

In this study, alpha and beta diversity are the main analysis methods used. Alpha diversity describes the species richness (number of taxonomical classifications) and evenness (the abundances of each classification) in a sample while beta diversity describes the differences between samples based on their species richness, evenness and/or phylogenetic distance (taxonomical relatedness). Both measures have several analysis methods that can be used to calculate their values. To compare the effects different alpha diversity methods have on results and conclusions, Shannon entropy, Simpson's index, bias-corrected Chao1 and Total number alpha diversity methods are analysed. From the beta diversity methods, Bray-Curtis and Jaccard are compared. Apart from alpha and beta diversity, there are also other ways to visualize and analyse microbial data. These include, for example, differential abundance and antibiotic resistance gene classification analyses which are also included in this study.

1.9.1 Alpha diversity methods

Shannon entropy takes both species richness and evenness into account with greater emphasis on the species richness. It is calculated by multiplying the percentage of a species in a sample with the logarithm of the inverse value of the percentage. The values of each species are then added together to create the final value called Shannon entropy (Thukral, 2017). The higher the Shannon entropy is the more diverse the sample is. However, as Shannon entropy takes both species richness and evenness into account, the same value can be reached with different taxonomical profiles which makes analysing diversity changes difficult.

Simpson's index also takes species richness and evenness into account but with a greater emphasis on evenness. It does this by dividing the sum of reads, OTUs or ASVs of each species squared with the total number of species squared (Thukral, 2017). The resulting value is always between 0 and 1. With the complemented value, the higher the index is the more diverse the sample is.

Bias-corrected Chao 1 only estimates the species richness of a sample. It takes rare taxa into account, which can help correct for low sampling and incomplete sequencing. However, it is limited to presence-absence data and thus cannot give information about species evenness in a sample. (Thukral, 2017) Similarly to Chao 1, the Total number method also only gives values based on the species richness of the sample. In fact, it only calculates how many species or taxonomical classifications there are in a sample.

1.9.2 Beta diversity methods

Bray-Curtis is one of the most commonly used beta diversity measures in microbiome analyses (McKnight et al., 2019). It quantifies dissimilarity between two samples to values between 0 and 1 with 1 meaning complete dissimilarity and 0 meaning complete similarity. Meanwhile, the Jaccard similarity index measures the diversity between samples by calculating how many species are shared by the samples and dividing this by the combined number of species in both samples. This results in a value between 1 and 0 with 1 meaning complete similarity and 0 meaning complete dissimilarity (Podani et al., 2013). In CLC Genomics Workbench, Jaccard is calculated as a dissimilarity index rather than a similarity index, so it gives the complemented value of the calculation.

To visualise the taxonomical differences between all samples, two- or three-dimensional principal coordinates analysis (PCoA) is used. The PCoA provides a summary of the beta diversities in a scatterplot format (Goodrich et al., 2014). The scatterplot can be used to identify compositionally similar and dissimilar samples based on their relative distances to each other. Similar compositions cluster together while dissimilar samples are further apart. This information can be utilised in recognising patterns in the dataset.

To ascertain the statistical significance of the observed patterns between different samples or sample groups, permutational multivariate analysis of variance (PERMANOVA) can be performed. This analysis provides a pseudo-f-statistic value, a p-value and a Bonferroni-corrected p-value. The pseudo-f-statistic describes the size of the observed difference with higher values meaning greater difference while the p-values describe the statistical significance of the observed difference. The Bonferroni-corrected p-value provides a more conservative value for the significance which takes the number of comparisons into account.

1.9.3 Differential abundance

Differential abundance analysis is used to identify microbes whose abundances differ statistically significantly between samples or sample groups. This information can then be used, for example, to conclude the roles of these microbes in specific conditions. In this study, differential abundance analysis is utilised to identify the bacteria that are the most affected by being frozen and differentially expressed due to gender.

1.9.4 ShortBRED

The Short, Better Representative Extract Dataset (ShortBRED) can be used to identify antibiotic resistance-causing genes from shotgun-sequenced data. The genes are identified based on short peptide sequences that are highly representative of specific protein families linked to antibiotic resistance. The use of these marker sequences should reduce the number of false positives as only relevant proteins are identified. (Kaminski et al., 2015)

2 Results

First, the qualities of the sequencings were checked by comparing the yield and the percentage of bases with a quality score of 30 or higher (%Q30) values to those expected according to the manufacturer (Appendix 1). Based on the values, both sequencings were successful. However, the %Q30 of shotgun sequencing was slightly lower than expected.

2.1 Taxonomical classification

After trimming and filtering, the 16S V3V4 rRNA sequenced reads were taxonomically classified with four different pipelines: OTU clustering with Greengenes reference database, OTU clustering with SILVA reference database and ASV assigning with SILVA reference database in CLC Genomics Workbench and in Chipster. Meanwhile, the shotgun-sequenced reads were classified by taxonomic profiling with the UHGG database. Additionally, human, viral and fungal reads were taxonomically profiled from the shotgun-sequenced samples to ascertain a more accurate estimate of the percentage of unclassifiable reads.

2.1.1 Quality of the taxonomical classification methods

In general, there were approximately 885,000 reads per sample received from 16S V3V4 rRNA sequencing (Figure 1A). Of these reads both Greengenes and SILVA OTU clustering were able to taxonomically classify approximately 305,000 reads per sample (34.5%). At least 55.3% of the reads were lost in trimming and filtering before OTU clustering. ASV assigning in CLC Genomics Workbench was able to taxonomically classify approximately 493,000 reads per sample (55.7%) while at least 40.3% of reads were lost in trimming and filtering. Lastly, ASV assigning in Chipster was able to taxonomically classify approximately 378,000 reads per sample (42.7%) while at least 52.6% of reads were lost in trimming and filtering. The last 10% or less of reads in each taxonomical classification method consisted most likely of unclassifiable reads. In summary, ASV assigning in CLC Genomics Workbench was able to taxonomically classify the most reads while both OTU clustering methods classified the lowest number of reads.

Even though the ASV assigning methods were able to classify more reads than the OTU clustering methods, the roles were reversed when the number of taxonomical classifications (OTUs and ASVs) were compared (Figure 1B). In general, Greengenes OTU clustering identified 1,100 OTUs per sample, SILVA OTU clustering identified 1,900 OTUs per sample, ASV assigning in CLC Genomics Workbench identified 830 ASVs per sample and ASV assigning in Chipster identified

590 ASVs per sample. Furthermore, when the total number of taxonomical classifications of the faecal samples reaching species level were compared, SILVA OTU clustering had a 47.8% success rate while ASV assigning in Chipster had a 31.9% success rate, Greengenes OTU clustering had a 30.4% success rate and ASV assigning in CLC Genomics Workbench had a 23.0% success rate when all unidentifiable, uncultured and other unclear classifications were removed. In summary, SILVA-based OTU clustering had the highest number of overall and species-level classifications.

The positive and negative controls of 16S V3V4 rRNA sequencing had approximately 410,000 and 4,900 reads and 323,000 and 3,600 taxonomical classifications in general respectively based on the SILVA OTU clustering. Compared to the mean read count of the faecal samples, the negative controls had at most 4.5% of reads (~13,800 reads) in the classifications.

On the other hand, after shotgun sequencing, there were 10 million reads per sample in general (Figure 1C). Of these reads 57% were taxonomically classified as bacteria with 59.3% of them being classified up to the species level. Of the remaining reads 0.02% were human, 0.7% were viruses, 0.0003% were fungi and 18.2% were unclassifiable reads. The remaining 23.7% of reads were lost during trimming and filtering.

With shotgun sequenced samples, the number of taxonomical classifications per faecal sample was in general approximately 340 for bacteria and 41 for viruses, while positive and negative controls had 91 and 200 bacteria, respectively (Figure 1D). The positive controls contained only a few bacteria (8-18), so it was not surprising that fewer bacteria were identified from them than from the negative control. The number of bacterial classifications in the negative control and the number of reads in these classifications (~7400 reads) were still lower than any of the faecal samples. Compared to the mean number of reads in bacterial taxonomical classifications of the faecal samples, the negative control had only 0.13% of reads in the classifications.

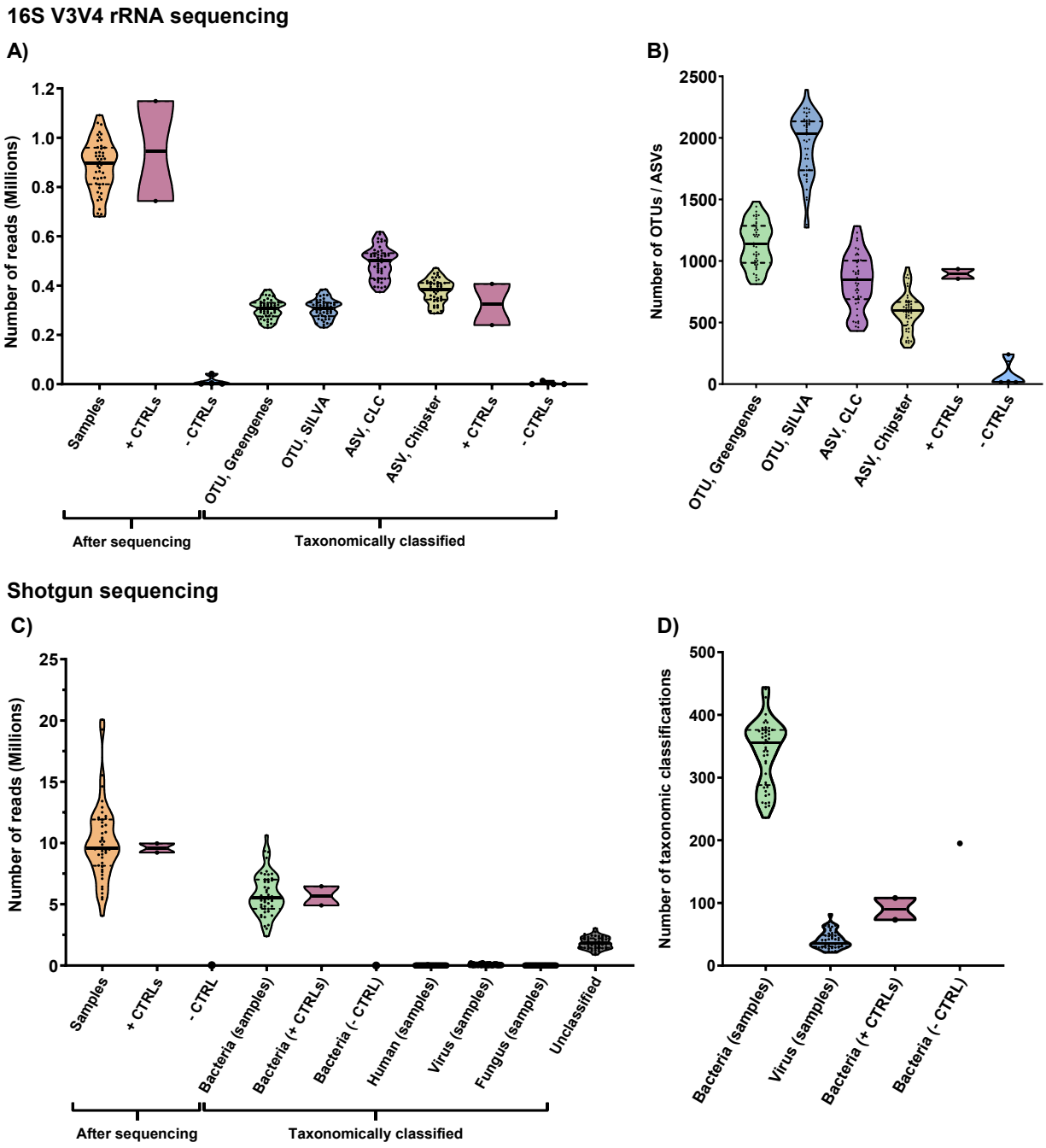


Figure 1. A) Distribution of the number of reads per sample after 16S V3V4 rRNA sequencing and different taxonomical classifications. The controls were OTU clustered with the SILVA reference database. B) Distribution of the number of OTUs or ASVs per sample according to the four different taxonomical classification methods; Greengenes and SILVA OTU clustering and SILVA ASV assigning in CLC Genomics Workbench and Chipster. The samples were sequenced with 16S V3V4 rRNA sequencing. The controls were OTU clustered with the SILVA reference database. C) Distribution of the number of reads per sample after shotgun sequencing and taxonomic profiling. D) Distribution of the number of bacterial and viral taxonomical classifications in shotgun-sequenced faecal samples and controls. Created in GraphPad Prism.

2.1.2 Most abundant bacteria

The four taxonomical classification methods of 16S V3V4 rRNA sequenced samples were compared to each other and to shotgun-sequenced samples by looking at the ten most abundant species-level taxonomical classifications of each method (Tables 1 and 2). Greengenes OTU clustering and ASV assigning in CLC Genomics Workbench had fewer species-level classifications than SILVA OTU clustering and ASV assigning in Chipster affecting the relevance of the most abundant species-level taxonomical classifications.

All four taxonomical classification methods used with 16S V3V4 rRNA sequenced samples had several classifications of *Bacteroides,* making it the most abundant genus. Of these, SILVA OTU clustering and ASV assigning in Chipster both identified *Bacteroides stercoris* and *Bacteroides vulgatus* from the faecal samples while Greengenes OTU clustering and ASV assigning in Chipster both identified *Bacteroides uniformis* and Greengenes OTU clustering and ASV assigning in CLC both identified *Bacteroides ovatus*. Apart from *Bacteroides*, these methods also had similarities with the other taxonomical classifications. SILVA OTU clustering and ASV assigning in Chipster both identified *Alistipes putredinis*, *Parabacteroides merdae* and *Phascolarctobacterium faecium* from the faecal samples while ASV assigning in CLC identified three different *Alistipes* and Greengenes OTU clustering identified *Parabacteroides distasonis.* Lastly, *Faecalibacterium prausnitzii* was identified by both Greengenes OTU clustering and ASV assigning in Chipster while *Faecalibacterium sp* was identified by SILVA OTU clustering.

When the ten most abundant taxonomical classifications of shotgun-sequenced and 16S V3V4 rRNA sequenced samples were compared, ASV assigning in Chipster identified five species and one genus, SILVA OTU clustering identified four species and two genera, Greengenes OTU clustering identified three species and one genus and ASV assigning in CLC identified two genera identically with shotgun sequencing and taxonomic profiling. In summary, ASV assigning in Chipster had the most accurate species-level taxonomical classifications when its ten most abundant species-level taxonomical classifications were compared with those from shotgun sequencing.

Table 1. Taxonomical classifications and percentages of the ten most abundant bacteria of 16S V3V4 rRNA sequenced samples at the species level.

The taxonomical classifications were identified with OTU clustering with Greengenes and SILVA reference databases and ASV assigning with SILVA reference database in CLC Genomics Workbench and Chipster programs. Genus and species classifications are used to describe the taxonomy. The taxonomical classifications are in alphabetical order so that closely related taxonomies are close to one another.

OTU (Greengenes)		**OTU (SILVA)**		**ASV (CLC)**		**ASV (Chipster)**	
%	**Taxonomy Genus species**	**%**	**Taxonomy Genus species**	**%**	**Taxonomy Genus species**	**%**	**Taxonomy Genus species**
0.5	*Collinsella aerofaciens*	4.2	*Bacteroides dorei*	0.01	*Bacteroides bacterium NLAE-zl-C585*	1.8	*Bacteroides coprocola*
0.7	*Bacteroides caccae*	1.5	*Bacteroides stercoris*	0.06	*Bacteroides ovatus*	2.0	*Bacteroides stercoris*
0.5	*Bacteroides fragilis*	2.4	*Bacteroides vulgatus*	0.03	*Bacteroides ovatus CL02T12C04*	3.0	*Bacteroides uniformis*
1.3	*Bacteroides ovatus*	1.2	*Bacteroides swine fecal bacterium RF3E-Xyl1*	0.2	*Bacteroides salyersiae*	3.1	*Bacteroides vulgatus*
3.3	*Bacteroides uniformis*	1.8	*Alistipes putredinis*	0.01	*Bacteroides sp. Marseille-P3132*	1.7	*Alistipes putredinis*
0.6	*Parabacteroides distasonis*	2.1	*Parabacteroides merdae*	0.1	*Coprobacter fastidiosus*	1.8	*Parabacteroides merdae*
0.6	*Blautia obeum*	2.4	*Agathobacter rectale M104/1*	0.2	*Alistipes inops*	1.7	*Blautia massiliensis*
6.7	*Faecalibacterium prausnitzii*	2.4	*Blautia obeum*	0.06	*Alistipes sp. cv1*	1.8	*Fusicatenibacter saccharivorans*
0.8	*[Eubacterium] biforme*	3.6	*Faecalibacterium sp*	0.06	*Alistipes bacteroides sp. DSM 12148*	4.5	*Faecalibacterium prausnitzii*
2.0	*Akkermansia muciniphila*	1.1	*Phascolarcto-bacterium faecium*	0.03	*Colidextribacter massiliensis*	1.4	*Phascolarcto-bacterium faecium*

The most abundant genus in the shotgun-sequenced faecal samples according to the taxonomic profiling was *Bacteroides* (Table 4). Other abundant genera (>1.9%) in the shotgun-sequenced samples were *Alistipes, Fusicatenibacter* and *Akkermansia.* On the phylum level, the ten most abundant taxonomical classifications were divided into three categories*: Bacteroidota* (5), *Firmicutes* (4) and *Verrumicrobiota* (1). On the family level, the *Firmicutes* were further divided into *Lachnospirales* (3) and *Oscillospirales* (1).

Table 2. Taxonomy of the ten most abundant bacterial species from shotgun sequencing.

The taxonomical classifications are given on genus and species level. The taxonomies are in order according to their taxonomical relatedness.

Taxonomical profiling	
%	**Taxonomy** **Genus species**
3.62	*Bacteroides stercoris*
3.63	*Bacteroides uniformis*
7.90	*Bacteroides_B dorei*
1.55	*Prevotella sp003447235*
2.35	*Alistipes putredinis*
1.72	*Agathobacter rectalis*
1.51	*Blautia_A wexlerae*
1.99	*Fusicatenibacter saccharivorans*
1.34	*Faecalibacterium prausnitzii_G*
1.91	*Akkermansia muciniphila*

The distribution of bacteria across all samples was visualized with bar charts. The samples had a high number of bacteria as 30 of the most abundant taxonomical classifications only covered 50-60% of all the bacteria (Figure 2). Each donor had an individual bacterial profile which showed similarly in all three sample types. The most abundant bacterial genus in the samples with both sequencing methods was *Bacteroides* as they covered eight of the 30 most abundant taxonomical classifications resulting in at least a 21% overall abundance. Other abundant bacterial genera with both sequencing methods were *Faecalibacterium, Alistipes, Blautia, Fusicatenibacter, Agathobacter* and *Akkermansia.* The abundances of specific genera and species varied from donor to donor.

On the phylum level, the 30 most abundant taxonomical classifications of 16S V3V4 rRNA sequenced and SILVA OTU clustered samples were divided into four sections so that *Bacteroidota* covered 12, *Firmicutes* covered 16, *Proteobacteria* covered one and *Verrumicrobiota* covered one

of the taxonomical classifications on the phylum level. The *Bacteroidota* were further divided into eight *Bacteroides*, one *Prevotella*, one *Alistipes* and one *Parabacteroides* on the genus level and the *Muribaculaceae* on the family level. The *Firmicutes* were further divided into one *Bacilli*, one *Negativicutes* and 14 *Clostridia* on the class level. The *Clostridia* then further divided into one *Christensenellales*, six *Oscillospirales* and seven *Lachnospirales* on the order level. The *Oscillospirales* were even further divided into one *Oscillospiraceae* and five *Ruminococcaceae* on the family level.

With shotgun sequencing the 30 most abundant taxonomical classifications were divided into four sections with *Bacteroidota* covering 17, *Firmicutes* covering 11 and *Verrumicrobiota* covering one of the taxonomical classifications on the phylum level. The *Bacteroidota* were further divided into eight *Bacteroides*, three *Alistipes,* two *Prevotella*, one *Parabacteroides* and one *UBA7173* on the genus level, while some *Bacteroidaceae* were only identified on the family level and *Bacteroidales* on the order level. The *Firmicutes* were all from the *Clostridia* class, which was further divided into one *Christensenellales*, four *Oscillospirales* and five *Lachnospirales* on the order level. The *Oscillospirales* were even further divided into one *Oscillospiraceae* and three *Ruminococcaceae* on the family level. The last of the four sections consisted of one kingdom-level taxonomical classification: *Bacteria.* The fact that such a low classification had a high abundance is probably due to overly general sequences or a lack of reference sequences.

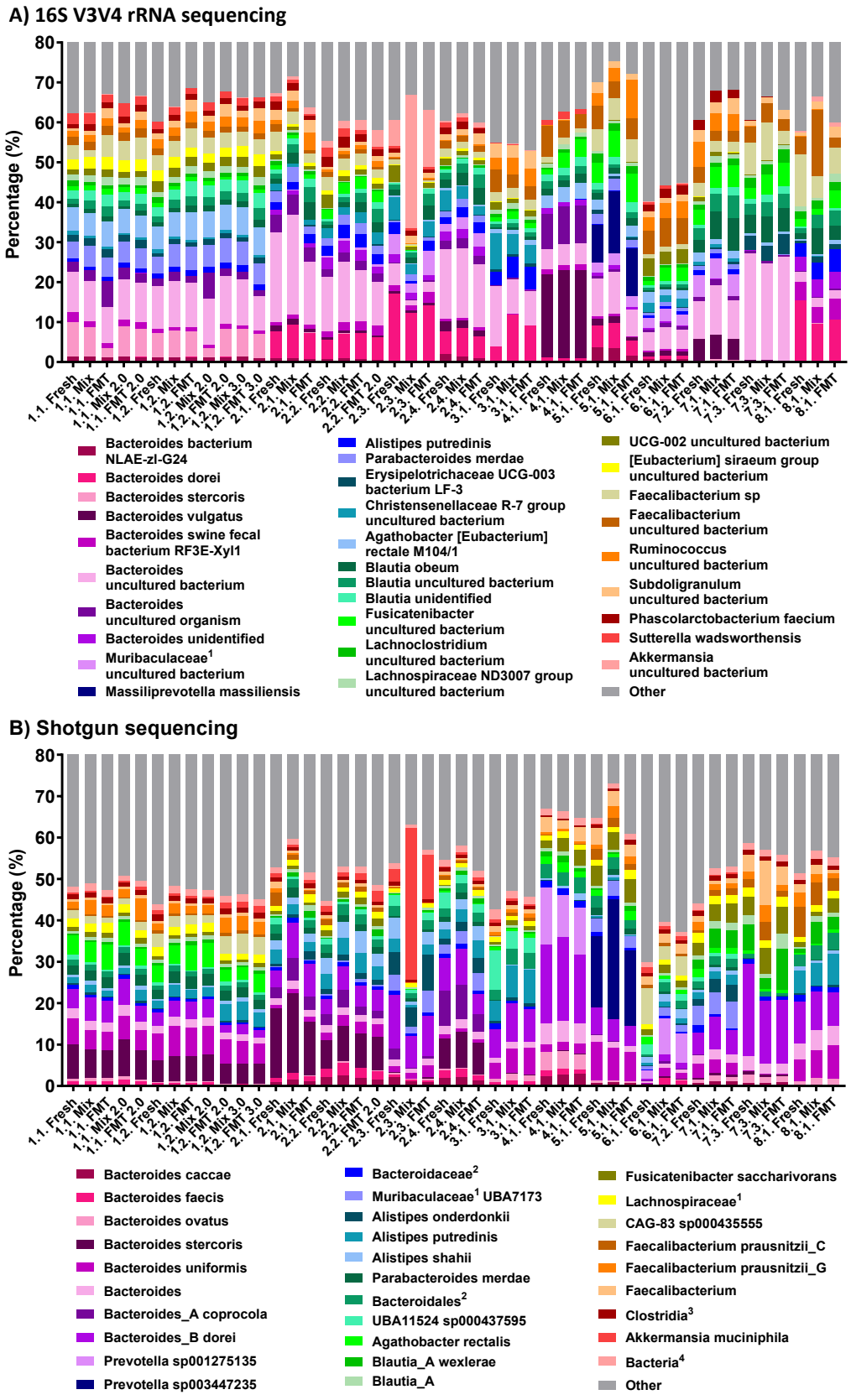


Figure 2. A) Bar chart of the 30 most abundant OTUs of the 16S V3V4 rRNA sequenced and SILVA OTU clustered faecal samples. B) Bar chart of the 30 most abundant taxonomical classifications of the shotgun-sequenced faecal samples. The classifications are named on genus and species level whenever possible, and they are in alphabetical order so that taxonomically closely related classifications are close to each other. Created in GraphPad Prism. [1] Family, [2] Order, [3] Class, [4] Kingdom

2.1.3 Positive and negative controls

In total, there were two positive controls used with both sequencing methods and four negative controls used with the 16S V3V4 rRNA sequencing one of which was also included in shotgun sequencing. The positive controls were the Zymo Gut Microbiome Standard which was a control for the DNA extraction and the Zymo Microbial Community DNA Standard which was a control for the PCR and library preparation. Three of the four negative controls in 16S V3V4 rRNA sequencing were controls for the DNA extraction while one controlled the PCR and library preparation. The three DNA extraction controls were lysis buffer, 10% glycerol and OMNIgene GUT solution. The lysis buffer was also included in shotgun sequencing as a negative control for the DNA extraction.

The Zymo Gut Microbiome Standard consists of 18 bacteria, one archaeon and two fungi with varying abundances. The fungi were not classified from either sequencing, so these were excluded from the comparisons. Additionally, five of the bacteria were different *E. coli* strains which could not be reliably identified from either sequencing method as the taxonomies disclosed by the manufacturer did not match the ones used in SILVA or UHGG reference databases. However, multiple *E. coli* strains were identified by both sequencing and taxonomical classification methods suggesting that both were able to correctly or equally incorrectly identify them, especially as some of the *Escherichia* strains identified by both sequencing methods matched with each other.

Of the remaining 13 bacteria, 16S V3V4 rRNA sequencing and SILVA OTU clustering were able to correctly identify 11 on the species level. The bacteria with the lowest abundances of 0.001-0.0001% (*Enterococcus faecalis* and *Clostridium perfringens*) were not identified from the control showing that the limit of detection was approximately 0.01% abundance. On the other hand, shotgun sequencing and taxonomic profiling were able to correctly identify 9 of the 13 bacteria on the species level. Three of the bacteria with the lowest abundances ranging between 0.01-0.0001% (*Salmonella enterica, E. faecalis* and *C. perfingens)* were not identified from the control showing that the detection limit was approximately 0.01% abundance. The fourth bacterium (*Fusobacterium nucleatum*) that was not identified on the species level was either identified as another closely related species or on the genus level.

The one archaeon (*Methanobrevibacter smithii*) was correctly identified on the species level by both sequencing and taxonomic profiling methods. The abundances of the microbes of the positive controls on genus level per sequencing method are shown in Figure 3A. Based on the abundances, *Roseburia*, *Bifidobacterium* and *Lactobacillus* were under-sequenced by both methods while

Fusobacterium and *Clostridioides* were under-sequenced by only shotgun sequencing. Otherwise, the abundances were close to the expected abundances or over them.

The Zymo Microbial Community DNA Standard consists of eight bacterial species with equal abundances. Of these, both sequencing and taxonomic profiling methods were able to correctly identify all on the species level. However, the 16S V3V4 rRNA sequencing and SILVA OTU clustering underestimated the abundance of *E. faecalis* or misclassified it on the species level. The abundances of the microbes of these positive controls on the genus level per sequencing method are shown in Figure 3B. Most of the abundances were close to the expected values or over them. The only classification that differed somewhat from the expected value was *Pseudomonas* in the 16S V3V4 sequenced and SILVA OTU clustered positive control.

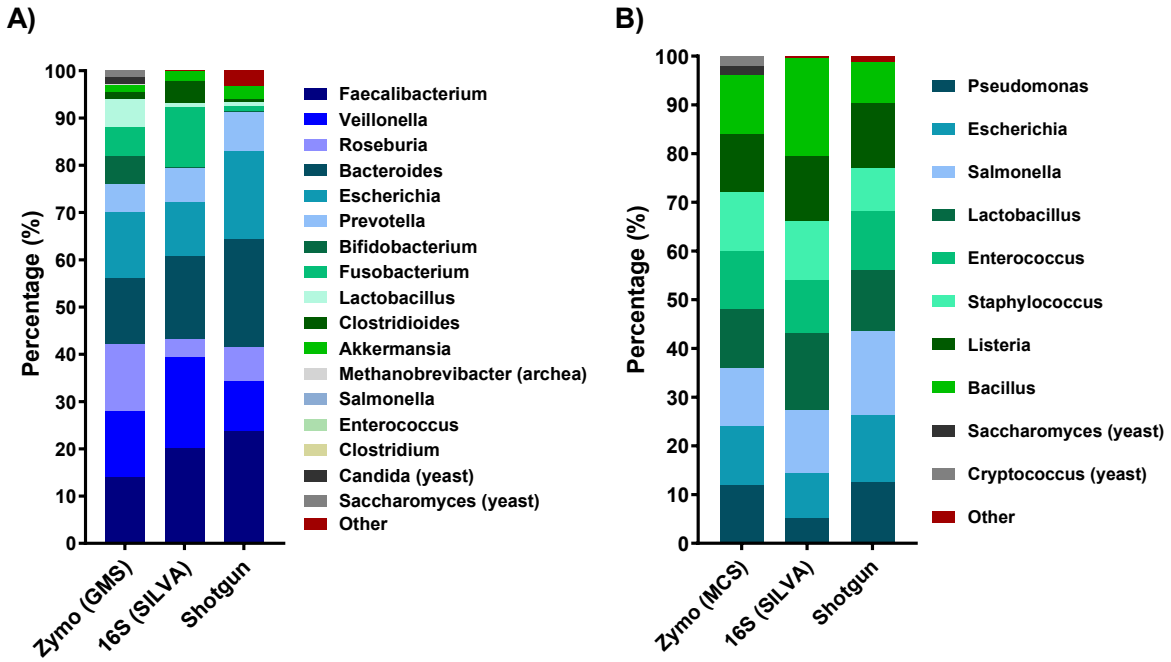


Figure 3. A) Zymo Gut Microbiome Standard's (GMS) microbes according to the manufacturer, 16S V3V4 rRNA sequencing with SILVA OTU clustering and shotgun sequencing with taxonomic profiling. B) Zymo Microbial Community Standard's (MCS) microbes according to the manufacturer, 16S V3V4 rRNA sequencing with SILVA OTU clustering and shotgun sequencing with taxonomic profiling. All profiles are shown on the genus level. Created in GraphPad Prism.

The 16S V3V4 rRNA sequenced and SILVA OTU clustered PCR and library preparation negative control identified only three genera from the sample. The lysis buffer control, on the other hand, identified the most bacteria of the negative controls. The most abundant genera in the Lysis, Glycerol and OMNIgene controls were *Blautia* (18.6%), *Fusicatenibacter* (12.2%), *Bacteroides* (11.6%), *Faecalibacterium* (8.4%) and *Escherichia-Shigella* (8.1%), while the most abundant genera of the PCR control were *Escherichia-Shigella* (65.7%), *Pseudomonas* (21.1%) and

Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium (13.2%). The most abundant genera of the shotgun-sequenced and taxonomically profiled negative lysis buffer control were *Blautia* (14.4%), *Bacteroides* (13.4%), *Fusicatenibacter* (11.2%), *Faecalibacterium* (7.4%) and *Bifidobacterium* (6.4%).

2.2 Alpha diversity

Four different alpha diversity metrics, Total number, Chao 1 bias-corrected, Simpson's index and Shannon entropy, were compared to each other based on the size and p-value of group-wise differences due to sample type, donors and gender (Tables 3 and 4). Of the four methods, Total number and Chao 1 bias-corrected were the most alike both visually and according to the sizes and p-values of the differences between groups. They both gave the highest overall p-values when comparing sample types and the lowest overall p-values when comparing donors and genders, while also giving the highest values for the size of the differences in all group-wise comparisons. On the other hand, Simpson's index was the opposite of the Total number and Chao 1 bias-corrected methods. It gave the lowest overall p-values when comparing sample types and the highest overall p-values when comparing donors and genders while giving the lowest values for the size of the differences in all group-wise comparisons. The low values for the size of the differences were due to Simpson's index being restricted to values between 0 and 1. The last alpha diversity method, Shannon entropy, gave values in between the other methods in general.

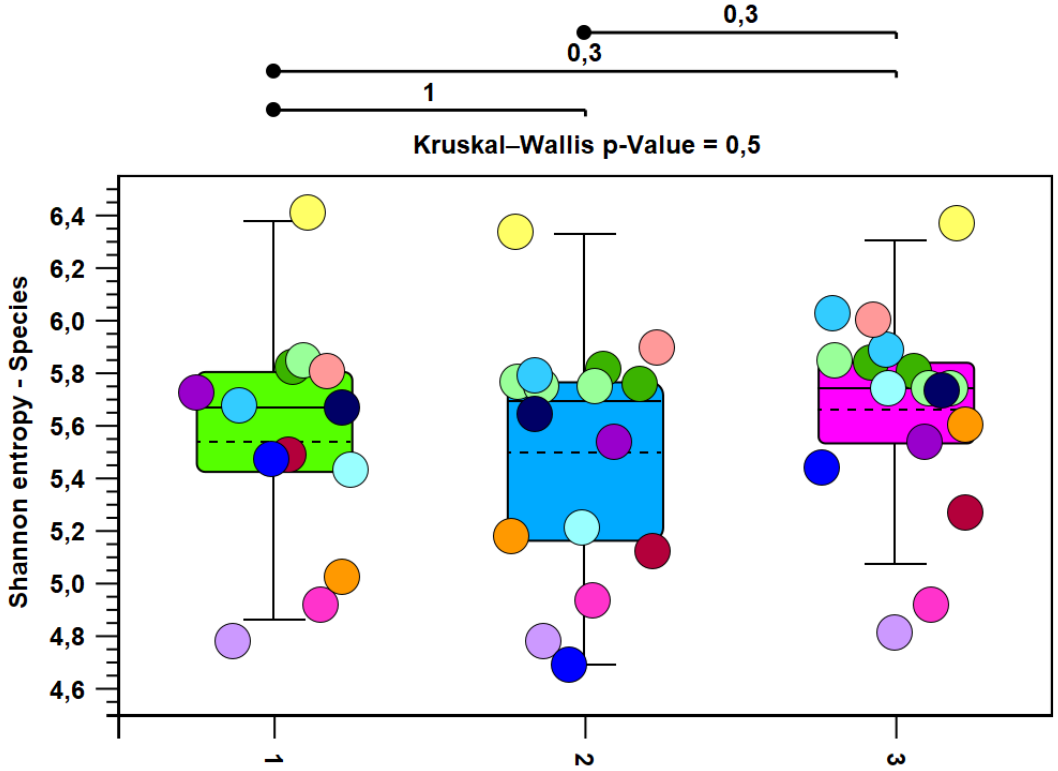
Table 3. Alpha diversity differences of 16S V3V4 rRNA sequenced and SILVA OTU clustered samples and shotgun-sequenced and taxonomically profiled samples by sample type.

Change and p-values between the means of the sample type groups as well as an overall Kruskal-Wallis p-value were calculated. Alpha diversity values were calculated with Total number, Chao 1 bias-corrected, Simpson's Index and Shannon entropy methods. Δ: Change between the means of sample type groups (females-males), Fresh: Fresh faecal samples, Mix: Faecal mixture samples, FMT: Stool transplant samples.

Alpha diversity by sample type		Fresh vs Mix		Fresh vs FMT		Mix vs FMT		Overall
		Δ	p-value	Δ	p-value	Δ	p-value	Kruskal-Wallis p-value
16S V3V4 rRNA	Total number	3.8	0.6	12.8	0.4	9.0	0.5	0.6
	Chao 1 Bias-corrected	3.7	0.8	12.9	0.4	9.3	0.6	0.7
	Simpson's Index	-0.004	0.9	0.008	0.2	0.01	0.1	0.2
	Shannon entropy	-0.05	1	0.1	0.3	0.2	0.3	0.5
Shotgun	Total number	-4.0	0.8	6.0	0.7	10.0	0.4	0.7
	Chao 1 Bias-corrected	-4.0	0.8	6.0	0.7	10.0	0.4	0.7
	Simpson's Index	-0.005	0.6	0.01	0.07	0.01	0.2	0.2
	Shannon entropy	-0.05	1	0.2	0.1	0.2	0.2	0.3

None of the alpha diversity methods found statistically significant differences between the sample types in either sequencing (overall p-values 0.2-0.7). The differences between fresh faecal samples and stool transplant samples were the largest (0.07-0.7) while the differences between fresh faecal samples and faecal mixture samples were the smallest (overall p-value 0.6-1) in general. The individual alpha diversity values aggregated on the species level by sample type and calculated with Shannon entropy are shown in Figure 4. Additionally, no statistically significant differences between the years the samples were frozen and sample types were observed. While some donors had differences between their faecal mixture and fresh faecal samples, these differences did not correlate with the time the samples were frozen for.

A) 16S V3V4 rRNA sequenced samples



B) Shotgun sequenced samples

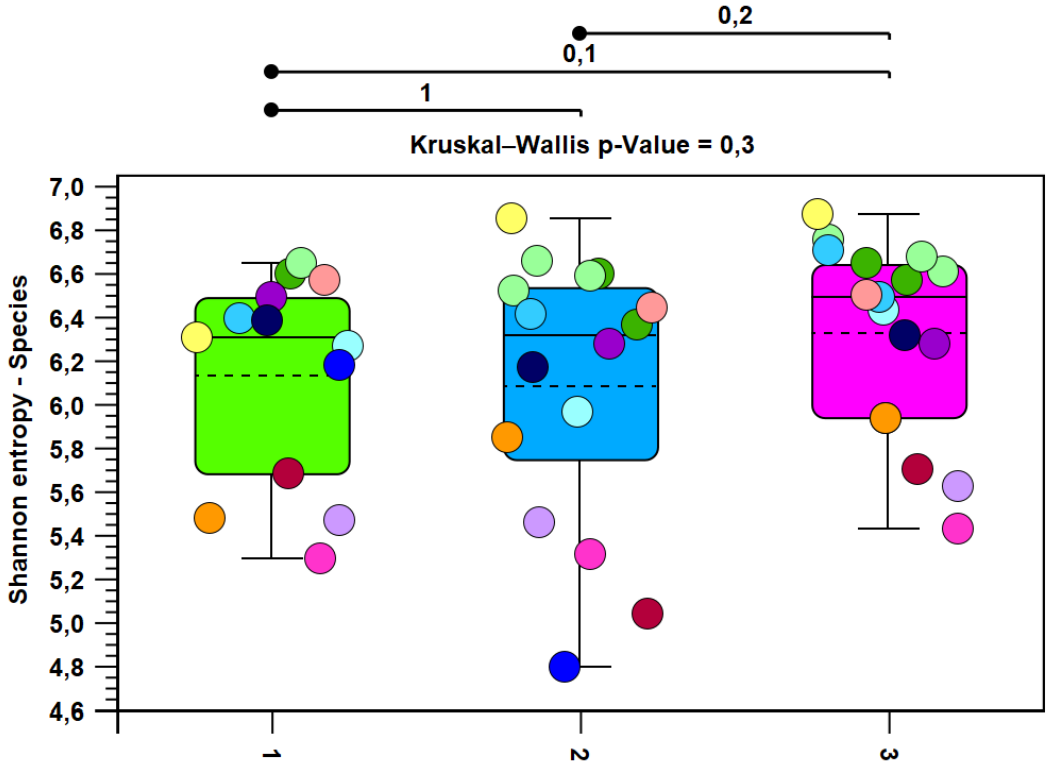


Figure 4. A) Alpha diversities of the 16S V3V4 rRNA sequenced and SILVA OTU clustered samples calculated by Shannon entropy on species level. B) Alpha diversities of the shotgun sequenced and taxonomically profiled samples calculated by Shannon entropy on the species level. The alpha diversity values are clustered by sample type (1-3) and the samples are coloured so that each donation has its own colour. Donations coloured with the same main colour such as green are all from the same donor. 1: Fresh faecal samples, 2: Faecal mixture samples, 3: Stool transplant samples

In donor and gender comparisons, all alpha diversity methods found statistically significant differences (Table 4). Some donors did not statistically significantly differ from all others but when they were considered as a whole, the donors did generally differ from each other. The individual alpha diversity values coloured by donation and aggregated on the species level by donors are shown in Figure 5. The figure shows how much alpha diversities of different donors and donations of a single donor differed from each other.

Donors 1, 2 and 7 all had multiple donations some of which differed from each other by their alpha diversity values. The donations of Donor 1, which were only a week apart, clustered closely together. Donor 2, on the other hand, had four donations in total with 4 days, 17.5 and 3.5 months apart. Although the samples did not cluster as closely as with Donor 1, they did stay relatively close to one another even though the variation was higher. Interestingly, the donations that were only four days apart (light blue dots in Figure 5) did not cluster together quite as closely as Donor 1's but were rather as far apart from each other as from the samples taken over a year later. The donations of Donor 7 were three months apart and showed a significant decrease in the alpha diversity of the faecal samples. In summary, it is very individualistic how much the species richness and/or evenness of the gut microbes changes over time.

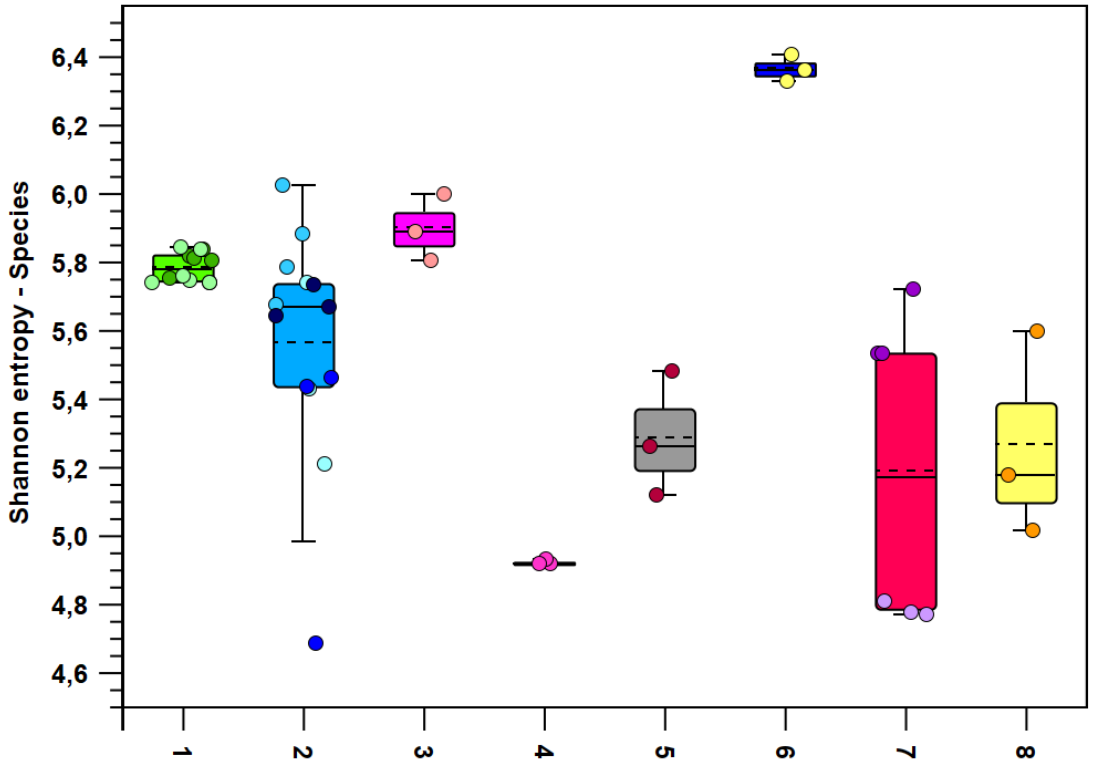
In the gender comparison, females had higher alpha diversity values than males (62-66 species more in general, p-value $4e^{-6}$-$5e^{-7}$) (Table 4). The individual alpha diversity values calculated with Shannon entropy and aggregated on the species level by gender are shown in Figure 6. Both genders had four donors, so the groups were evenly matched. Based on the alpha diversity values, females had a higher species richness and evenness than males in general.

Table 4. Alpha diversity differences of 16S V3V4 rRNA sequenced and SILVA OTU clustered samples and shotgun-sequenced and taxonomically profiled samples by donor and gender.

Change and p-values between the means of gender groups as well as an overall Kruskal-Wallis p-value for donor differences were calculated. Alpha diversity values were calculated with Total number, Chao 1 bias-corrected, Simpson's Index and Shannon entropy methods. ∆: Change between the means of gender groups (females-males), F: Female samples, M: Male samples

Alpha diversity		Donor	Gender	
		Kruskal-Wallis p-value	∆	p-value
16S V3V4 rRNA	Total number	1e-5	62.2	6e-7
	Chao 1 Bias-corrected	3e-5	62.2	4e-6
	Simpson's Index	0.0006	0.02	0.002
	Shannon entropy	5e-5	0.6	6e-6
Shotgun	Total number	4e-6	65.7	5e-7
	Chao 1 Bias-corrected	4e-6	65.7	5e-7
	Simpson's Index	0.0006	0.02	0.0003
	Shannon entropy	5e-5	0.7	5e-6

A) 16S V3V4 rRNA sequenced samples



B) Shotgun sequenced samples

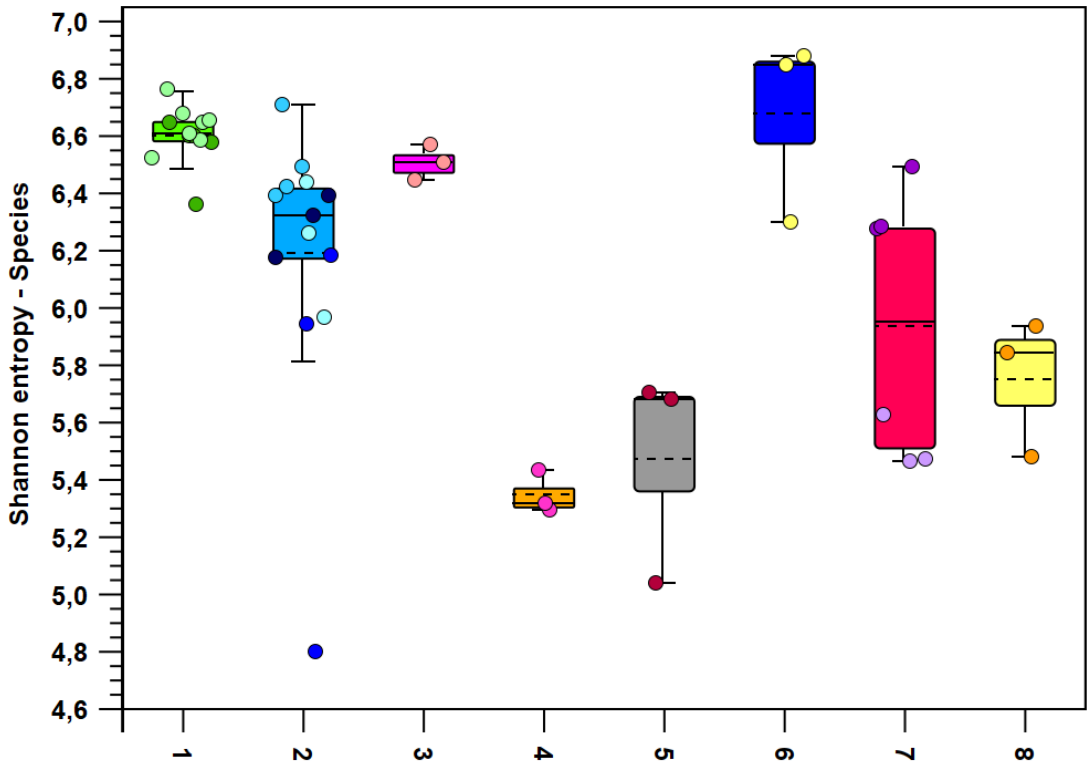
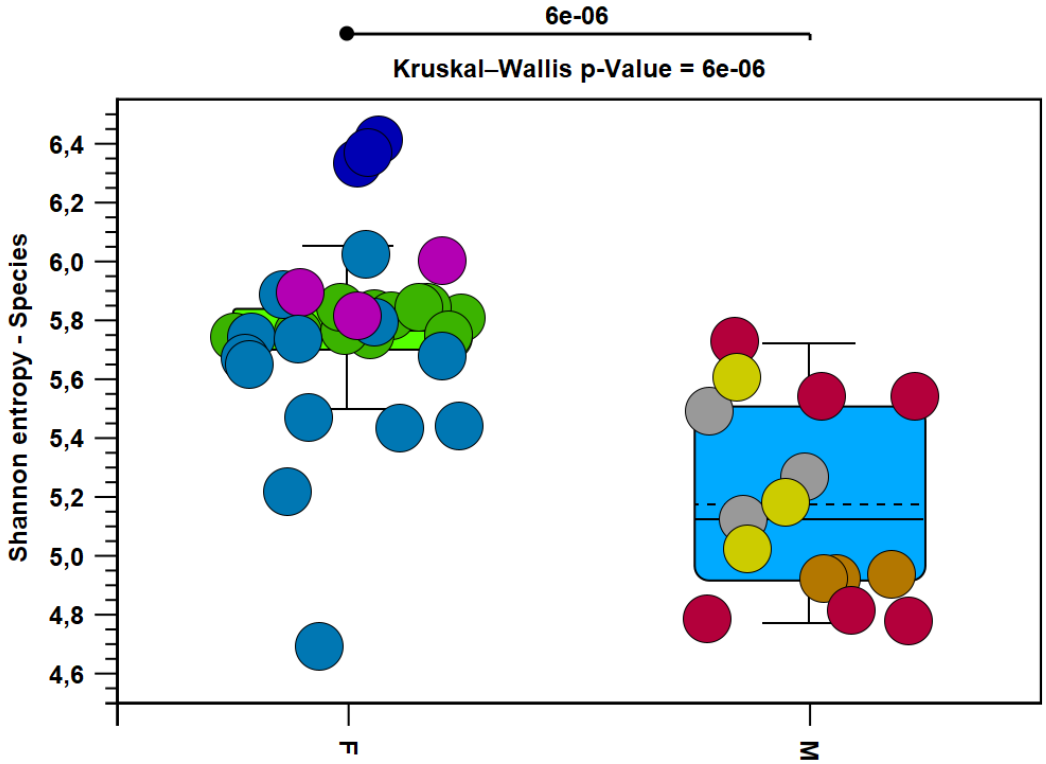


Figure 5. A) Alpha diversities of the 16S V3V4 rRNA sequenced and SILVA OTU clustered samples calculated by Shannon entropy on the species level. B) Alpha diversities of the shotgun sequenced and taxonomically profiled samples calculated by Shannon entropy on the species level. The alpha diversity values of the samples are clustered by donor (1-8) while the samples are coloured by donation.

A) 16S V3V4 rRNA sequenced samples



B) Shotgun sequenced samples

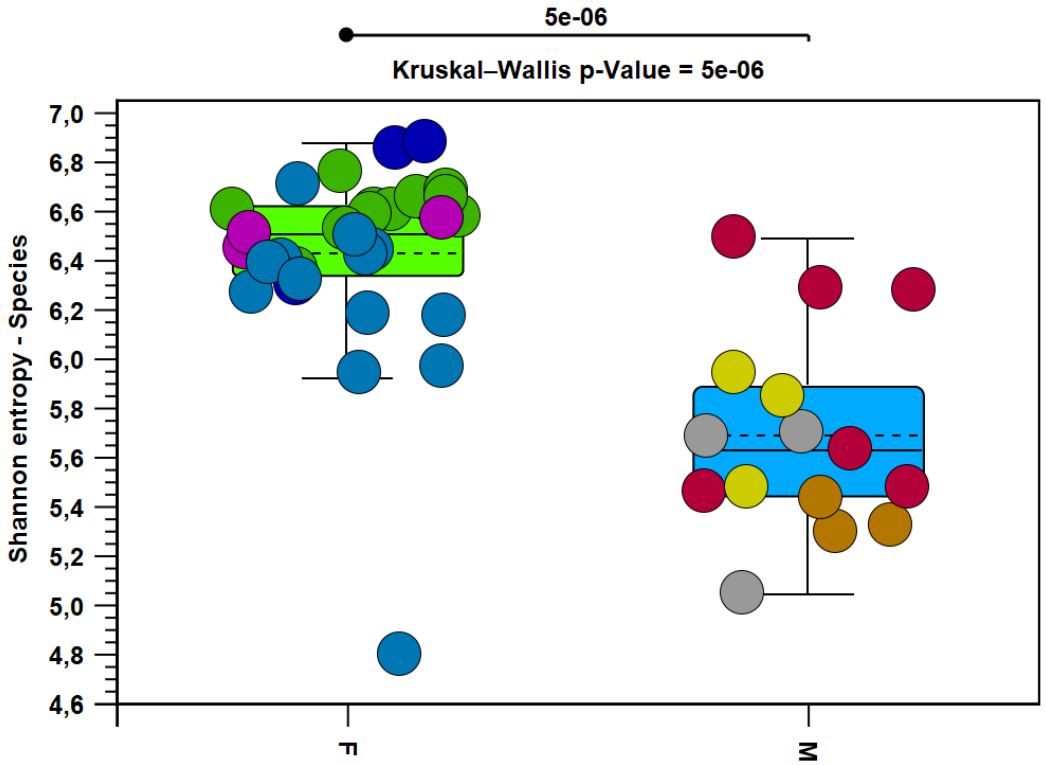


Figure 6. A) Alpha diversities of the 16S V3V4 rRNA sequenced and SILVA OTU clustered samples calculated by Shannon entropy on the species level. B) Alpha diversities of the shotgun sequenced and taxonomically profiled samples calculated by Shannon entropy on the species level. The alpha diversity values of the samples are aggregated based on the gender of each donor, and the samples are coloured by the donor. F: Female samples, M: Male samples.

2.3 Beta diversity

Jaccard and Bray-Curtis gave highly similar results from all three (sample type, donor and gender) group comparisons (Tables 5 and 6). However, in sample type comparisons Jaccard estimated the differences (16S: overall pseudo-f-statistic 0.523 vs 0.511, Shotgun: overall pseudo-f-statistic 0.501 vs 0.445) and their significances (16S: overall p-value 0.987 vs 0.964, Shotgun: overall p-value 0.998 vs 0.992) slightly higher than Bray-Curtis, while Bray-Curtis estimated higher differences between the donors (16S: overall pseudo-f-statistic 18.5 vs 12.2, Shotgun: overall pseudo-f-statistic 13.3 vs 8.7) and genders (16S: pseudo-f-statistic 8.7 vs 6.8, Shotgun: pseudo-f-statistic 11.7 vs 8.2) than Jaccard.

Similarly to alpha diversities, beta diversities showed no statistically significant differences between sample types (pseudo-f-statistic ~0.5, overall p-values 0.864-0.998, Bonferroni p-values 1) (Table 5) while donors (pseudo-f-statistic ~15, overall p-values 0.00001) and genders (pseudo-f-statistic ~8, p-values 0.00001-0.00006) did differ statistically significantly (Table 6). Consequently, the variance between sample type groups was lower than the variance within these groups while the opposite was true for the donors and genders.

The beta diversities by donors and gender were visualized with 2D PCoA scatterplots (Figures 7 and 8). The visualization showed clear clustering of samples due to their donor with donors 1 and 2 being the most different compared to the others (Figure 7). The samples also clustered based on gender (Figure 8). No clear clustering based on the time the samples were frozen was observed.

Table 5. Differences of the beta diversity values of 16S V3V4 rRNA sequenced and SILVA OTU clustered samples and shotgun sequenced and taxonomically profiled samples by sample type.

Pseudo-f-statistic describes the size of the difference between samples while the p-value describes the statistical significance of that difference. These values are shown for pairwise comparisons of each sample type group and the overall difference. Bray-Curtis and Jaccard methods were used to calculate the beta diversities and PERMANOVA analysis was used to calculate the differences between these values and their significance.

Beta diversity by sample type		Fresh vs. Mix		Fresh vs. FMT		Mix vs. FMT		Overall	
		Pseudo-f-statistic	p-value	Pseudo-f-statistic	p-value	Pseudo-f-statistic	p-value	Pseudo-f-statistic	p-value
16S V3V4 rRNA	Bray-Curtis	0.46	0.94	0.77	0.66	0.32	0.98	0.511	0.964
	Jaccard	0.50	0.96	0.72	0.77	0.36	0.99	0.523	0.987
Shotgun	Bray-Curtis	0.48	0.94	0.57	0.86	0.29	0.99	0.445	0.992
	Jaccard	0.55	0.96	0.60	0.92	0.36	1.00	0.501	0.998

Table 6. Differences of the beta diversity values of 16S V3V4 rRNA sequenced and SILVA OTU clustered samples and shotgun-sequenced and taxonomically profiled samples by donor and gender.

Pseudo-f-statistic describes the size of the difference between samples while the p-value and Bonferroni-corrected p-value describe the statistical significance of that difference. These values are shown for pairwise comparisons of genders and the overall difference of donors. Bray-Curtis and Jaccard methods were used to calculate the beta diversities and PERMANOVA analysis was used to calculate the differences between these values and their significance.

Beta diversity		Donor		Gender		
		Pseudo-f-statistic	p-value	Pseudo-f-statistic	p-value	Bonferroni p-value
16S V3V4 rRNA	Bray-Curtis	18.5	0.00001	8.7	0.00001	0.00001
	Jaccard	12.2	0.00001	6.8	0.00001	0.00001
Shotgun	Bray-Curtis	13.3	0.00001	11.7	0.00001	0.00001
	Jaccard	8.7	0.00001	8.2	0.00001	0.00001

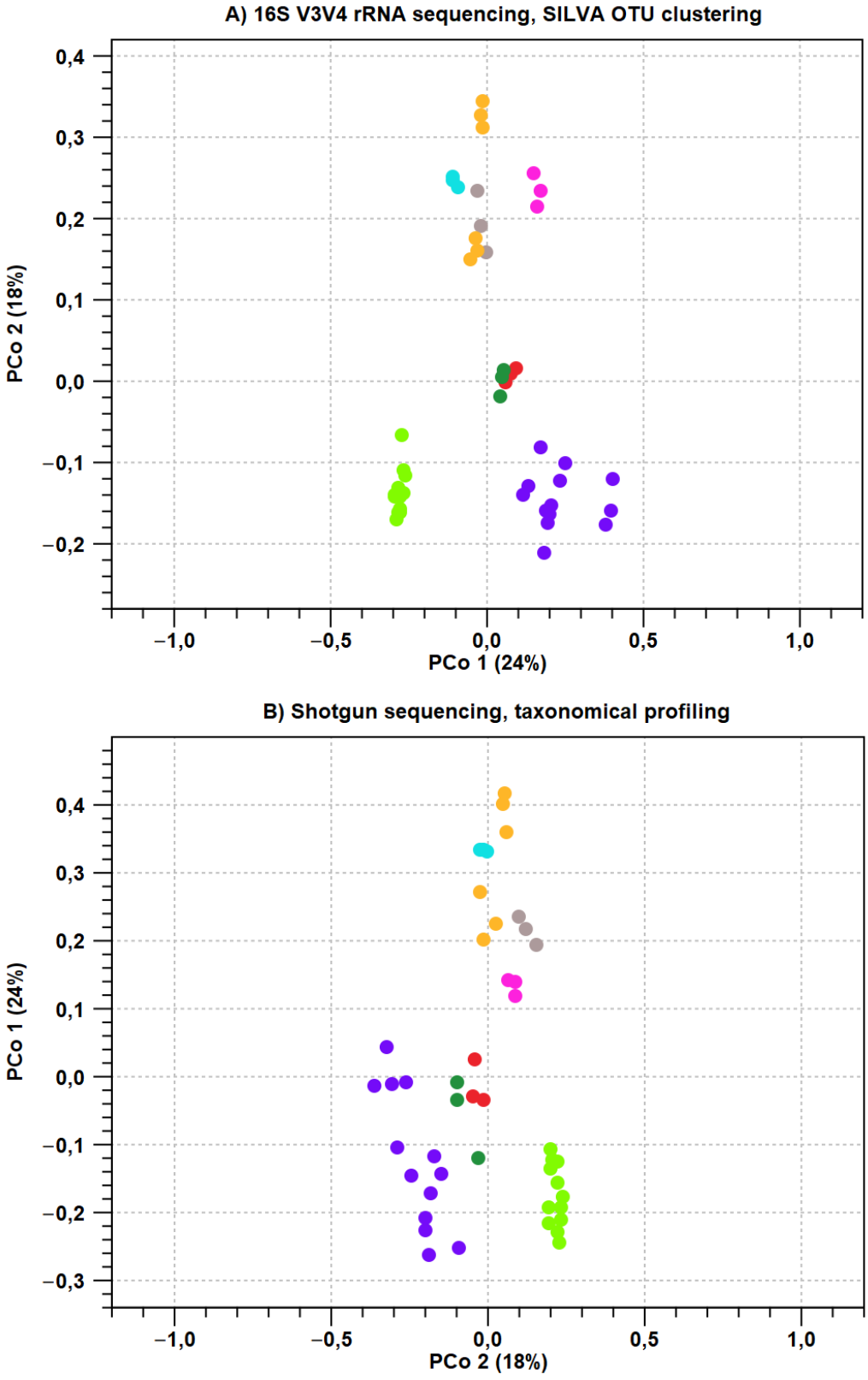


Figure 7. A) 2D PCoA scatterplot of the beta diversity values of 16S V3V4 rRNA sequenced and SILVA OTU clustered samples based on the Bray-Curtis calculation. B) 2D PCoA scatterplot of the beta diversity values of shotgun sequenced and taxonomically profiled samples based on the Bray-Curtis calculation. Light green: Donor 1, Purple: Donor 2, Red: Donor 3, Turquoise: Donor 4, Grey: donor 5, Dark green: Donor 6, Orange: Donor 7, Pink: Donor 8.

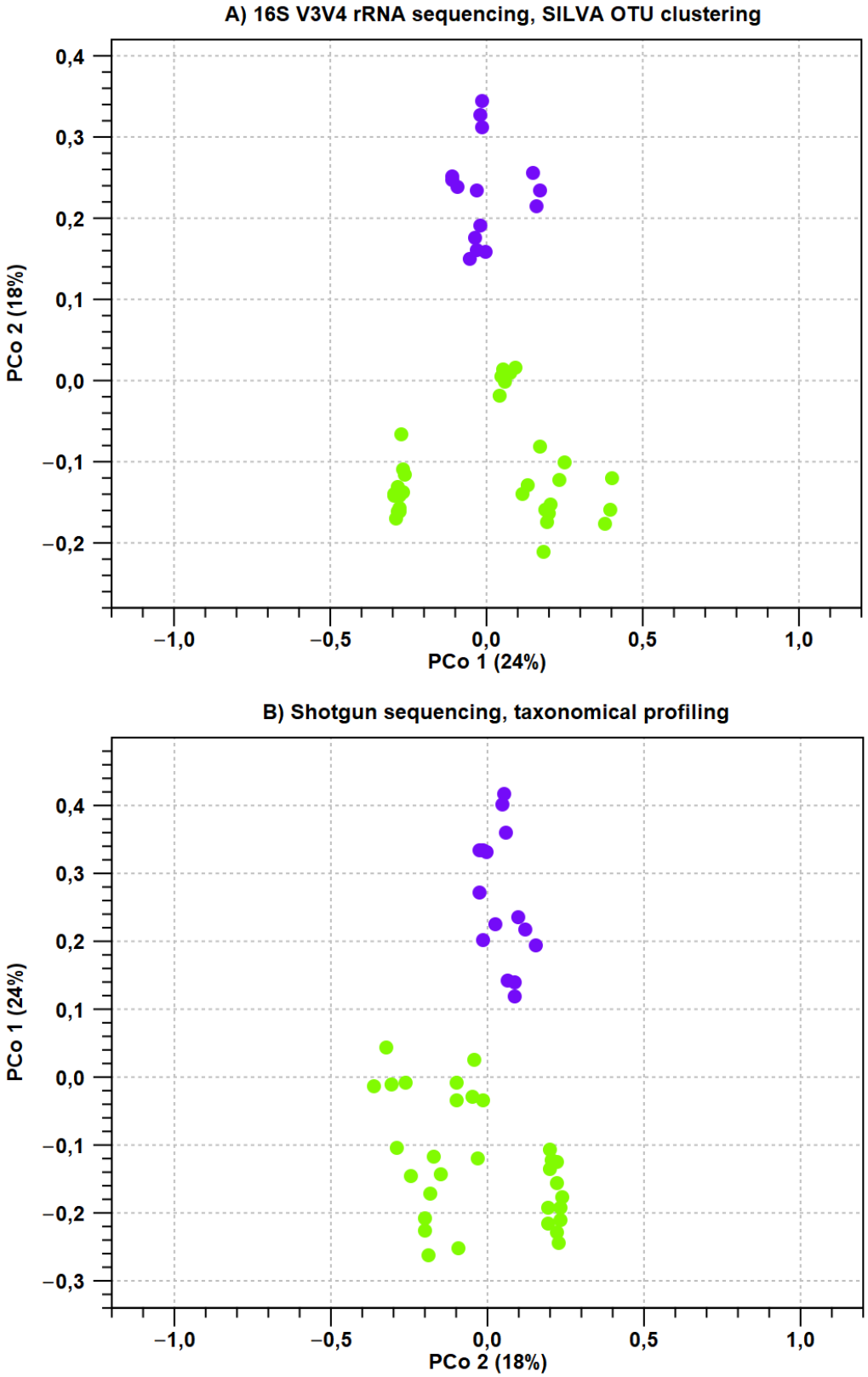


Figure 8. A) 2D PCoA scatterplot of the beta diversity values of 16S V3V4 rRNA sequenced and SILVA OTU clustered samples based on the Bray-Curtis calculation. B) 2D PCoA scatterplot of the beta diversity values of shotgun sequenced and taxonomically profiled samples based on the Bray-Curtis calculation. Beta diversity values of the samples are coloured by the gender of each donor so that green represents females and purple represents males.

2.4 Differential abundance

Differential abundances of bacterial taxa on the genus level between fresh faecal samples and faecal mixture samples were calculated to determine which bacteria were the most affected by being frozen at -80°C. In 16S V3V4 rRNA sequenced and SILVA OTU clustered samples, there were only eight statistically significant differentially abundant bacterial genera according to the false discovery rate (FDR) p-value. Seven of these were more abundant in the faecal mixture samples than fresh faecal samples while one was less abundant. The one genus that's abundance decreased after being frozen was *Cellulosilyticum* from the *Lachnospiraceae* family while *Peptoniphilus, Fenollaria, Anaerococcus, Prevotella, Prevotella_9, Porphyromonas* and *Varibaculum* increased in abundance after being frozen (Figure 9A).

In shotgun sequenced and taxonomically profiled samples, there were 42 statistically significant differentially abundant bacterial genera according to the FDR p-value. Of these, the twenty most statistically significant are shown in Figure 9B. Of the twenty most significant changes, seven increased and thirteen decreased in abundance after being frozen. The bacteria that increased were *CAG-312, Veillonellales, UBA10281, Catenibacterium, Porphyromonas, Libanicoccus* and *Pauljensenia,* while *CAG-239, UBA1822, UBA4068, TANB77, Pygmaiobacter, Phocea, Monoglobaceae, UBA5394, Streptococcaceae, Granulicatella, Erysipelotrichales, Gastranaerophilus* and *Gastranaerophilales* decreased in abundance after being frozen.

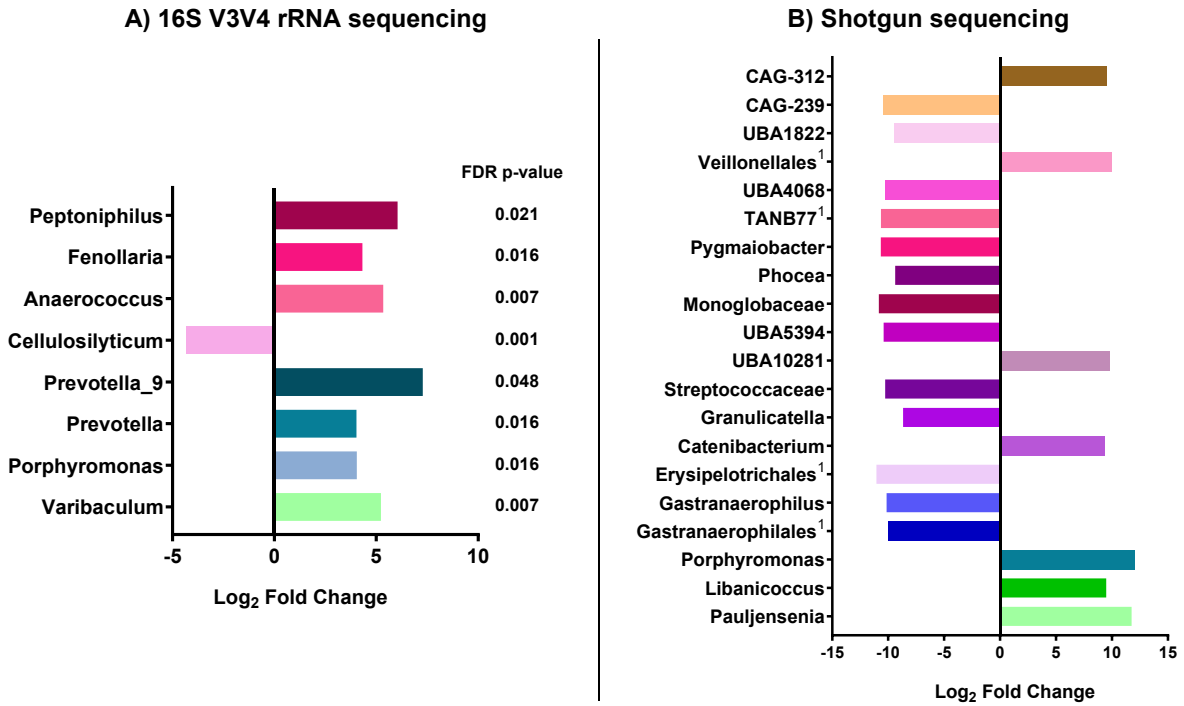


Figure 9. A) All statistically significant differentially abundant bacterial taxa between fresh and faecal mixture samples at genus level of the 16S V3V4 rRNA sequenced and SILVA OTU clustered samples. The significance of each difference is shown with the false discovery rate (FDR) p-value. The bacteria are coloured so that green represents *Actinobacteriota*, blue represents *Bacteroidota* and red represents *Firmicutes* phyla. B) 20 of the most statistically significant differentially abundant bacterial taxa between fresh and faecal mixture samples of the shotgun sequenced and taxonomically profiled samples. The taxa are named on the species level whenever possible. All of these differences had FDR p-values <0.0001. The bacteria are coloured so that green represents *Actinobacteriota*, greenish blue represents *Bacteroidota*, blue represents *Cyanobacteria,* purple, red and pink represent *Firmicutes*, orange represents *Proteobacteria* and brown represents *Verrumicrobiota* phyla. [1] Family-level identification.

The differential abundance of bacteria due to gender on the species level was also examined (Figure 10). Based on the phyla of the 20 most differentially expressed bacteria, females had more *Firmicutes* and *Bacteroides* while males had more *Proteobacteria* in general. On the genus and species level, males had higher abundances of *Bacteroides fragilis*, *Massiliprevotella massiliensis, Phascolarctobacterium, Parasutterella, Haemophilus uncultured bacterium* and *Haemophilus parainfluenzae T3T1* according to the 16S sequencing and SILVA OTU clustering while Shotgun sequencing and taxonomical profiling showed that males had a higher abundance of *Prevotella sp003447235, Lachnospira sp000436475, Parasutterella sp000980495* and *Haemophilus_D.* In total, 588 and 448 differentially abundant species were identified from 16S and shotgun sequencing, respectively.

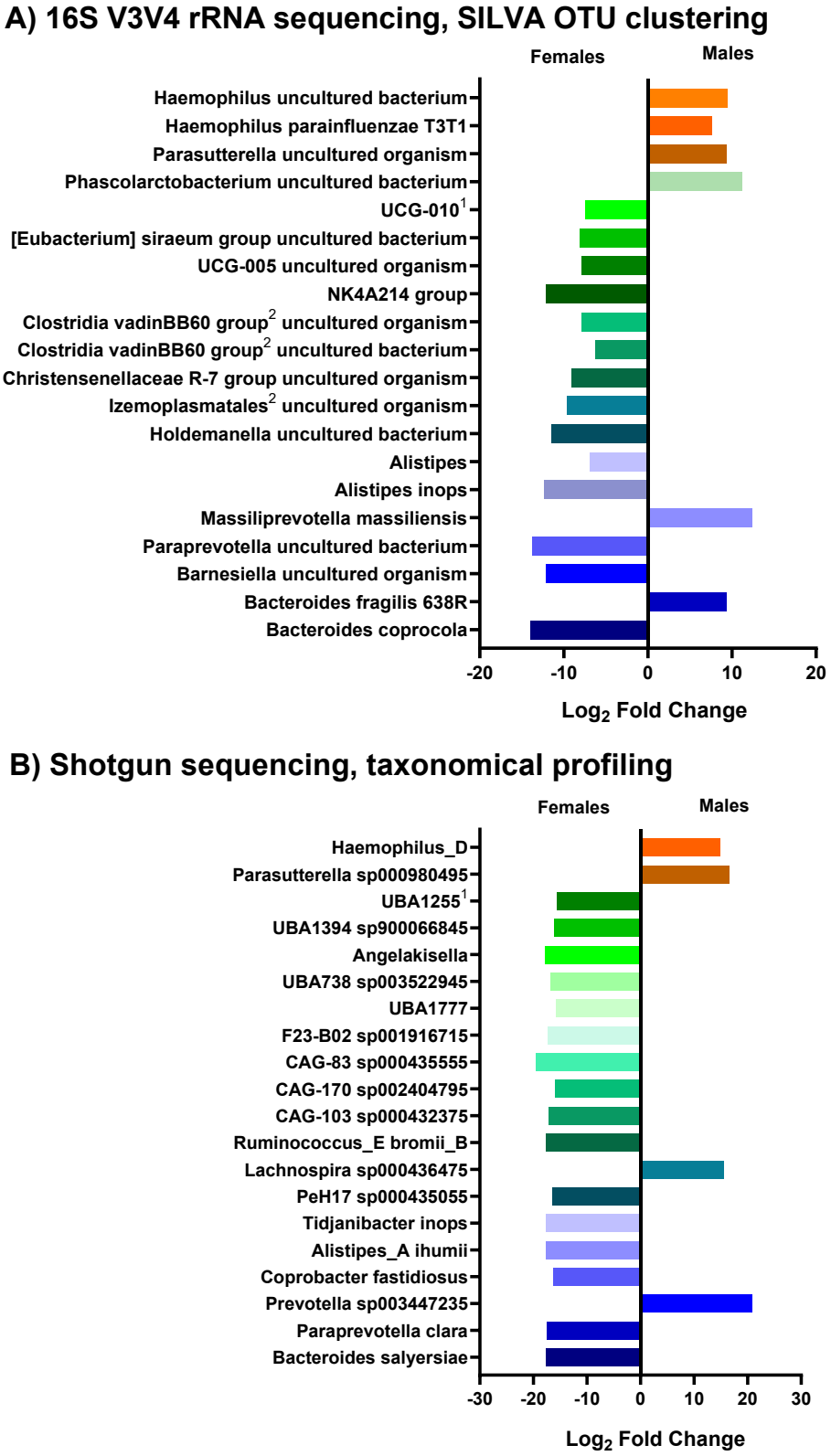


Figure 10. A) 20 most statistically significant differentially abundant bacterial taxa between genders of the 16S V3V4 rRNA sequenced and SILVA OTU clustered samples. B) 20 most statistically significant differentially abundant bacterial taxa between genders of the shotgun sequenced and taxonomically profiled samples. The taxa are named on the species level whenever possible. All of the differences had statistically significant false discovery rate (FDR) p-values (<0.0001). The bacteria are coloured so that blue represents Bacteroidota, green represents Firmicutes and orange represents Proteobacteria. [1] Family-level identification, [2] Order-level identification.

2.5 Antimicrobial resistance

The antibiotic resistance genes of the faecal samples were identified with ShortBRED and visualised with a bar chart of the 20 most abundant genes (Figure 11). Most of the samples did not have a lot of antibiotic resistance as the 20 most abundant genes covered approximately 90% of all of the identified antibiotic resistance genes. An exception to this was Donor 7's donation 7.3 which had a more diverse composition of antibiotic resistance genes than any of the other donations as the 20 most abundant genes only covered approximately 30% of the antibiotic resistance genes of the donation.

Antimicrobial resistance was most common against tetracyclines. In most samples, approximately 80% of the resistance genes conferred resistance to them (Table 7). Other common antibiotics that the bacteria were resistant to included macrolide antibiotics (7.02%) such as erythromycin.

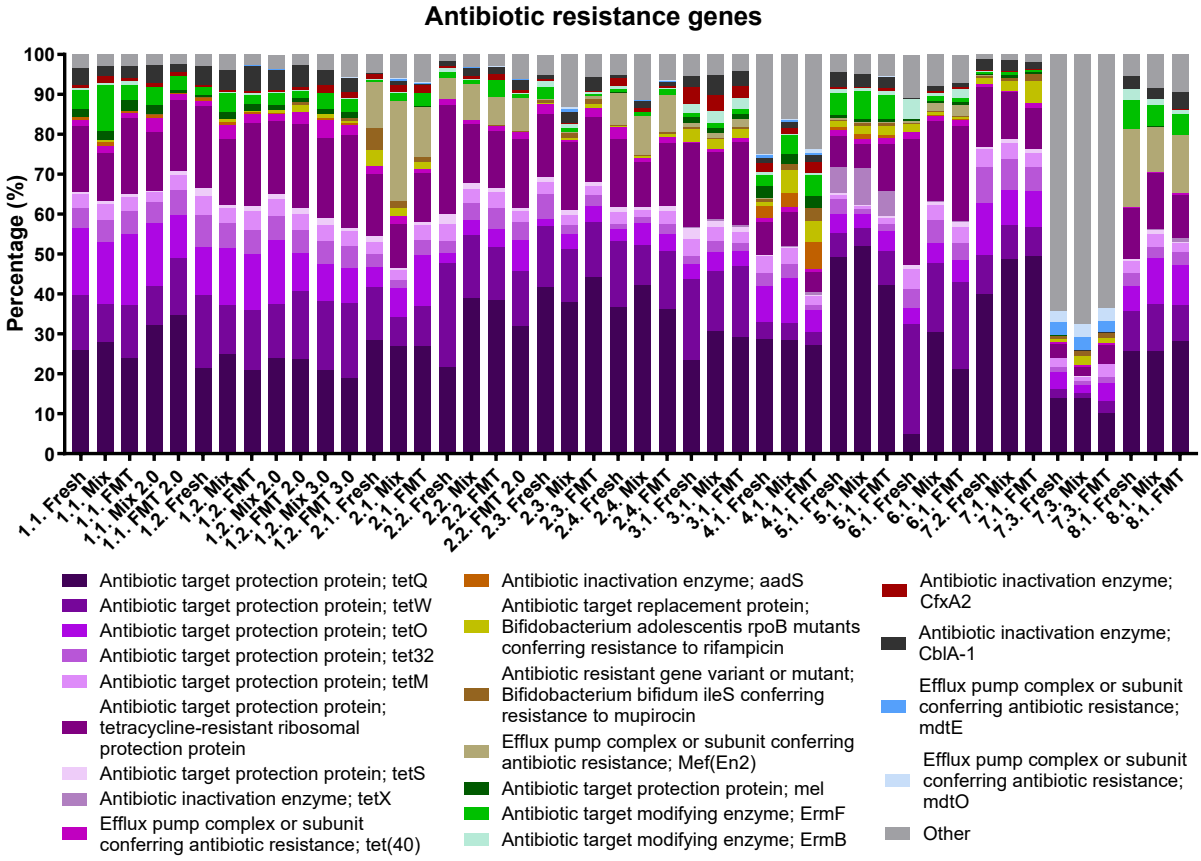


Figure 11. Bar chart of the 20 most abundant antibiotic resistance genes across all shotgun sequenced samples. The classifications of the genes are coloured according to the antibiotics they confer resistance to so that purple colours indicate resistance to tetracyclines, green colours indicate resistance to erythromycin and blue colours indicate resistance to antibiotic molecules. Other colours have individual antibiotics they are resistant to. The analysis was performed with ShortBRED in CLC Genomics Workbench. Created in GraphPad Prism.

Table 7. 20 of the most abundant antibiotic resistance genes and the antibiotics they confer resistance to across all shotgun-sequenced samples.

The classification of the gene, its overall abundance and the antibiotics it is resistant to are shown. The analysis was done with ShortBRED.

%	Classification	Confers Resistance To
29.2	Antibiotic target protection protein; tetQ	Tetracycline, doxycycline, minocycline, chlortetracycline, demeclocycline, oxytetracycline, tetracycline antibiotic
11.0	Antibiotic target protection protein; tetW	
7.3	Antibiotic target protection protein; tetO	
3.7	Antibiotic target protection protein; tet32	
3.1	Antibiotic target protection protein; tetM	
0.8	Antibiotic target protection protein; tetS	
0.8	Antibiotic inactivation enzyme; tetX	Tigecycline, tetracycline, doxycycline, minocycline, chlortetracycline, demeclocycline, oxytetracycline, tetracycline antibiotic
13.6	Antibiotic target protection protein; tetracycline-resistant ribosomal protection protein	Tetracycline antibiotic
1.4	Efflux pump complex or subunit conferring antibiotic resistance; tet(40)	Tetracycline
0.5	Antibiotic inactivation enzyme; aadS	Streptomycin, aminoglycoside antibiotic
1.3	Antibiotic target replacement protein; Bifidobacterium adolescentis rpoB mutants conferring resistance to rifampicin	Rifamycin antibiotic
0.7	Antibiotic resistant gene variant or mutant; Bifidobacterium bifidum ileS conferring resistance to mupirocin	Mupirocin
3.0	Efflux pump complex or subunit conferring antibiotic resistance; Mef(En2)	Macrolide antibiotic
0.8	Antibiotic target protection protein; mel	Erythromycin, telithromycin
2.6	Antibiotic target modifying enzyme; ErmF	Erythromycin, roxithromycin, lincomycin, telithromycin, clarithromycin, clindamycin, tylosin, spiramycin, azithromycin, dirithromycin, pristinamycin IA, quinupristin, pristinamycin IIA, madumycin II, griseoviridin, dalfopristin, pristinamycin IB, virginiamycin S2, vernamycin B-gamma, vernamycin C, patricin A, patricin B, ostreogrycin B3, oleandomycin, macrolide antibiotic, lincosamide antibiotic, streptogramin antibiotic
0.7	Antibiotic target modifying enzyme; ErmB	
0.9	Antibiotic inactivation enzyme; CfxA2	Cephamycin
2.3	Antibiotic inactivation enzyme; CblA-1	Cephaloridine, cephalosporin
0.6	Efflux pump complex or subunit conferring antibiotic resistance; mdtE	Antibiotic molecule
0.5	Efflux pump complex or subunit conferring antibiotic resistance; mdtO	Antibiotic molecule

2.6 Bacterial cultures

One donor's stool transplant sample was cultured on blood, lactose, CHROMagar orientation and Fastidious Anaerobe Agar (FAA) agar plates to check the viability of the bacteria (Appendix 3). Pure cultures were done from all unique colonies and their taxonomies were identified with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) from the pure cultures (Table 8). The blood, lactose and CHROMagar orientation plates mostly had *Escherichia coli* with one exception of *Bacillus amyloliquefaciens* while the colonies on FAA plates had more variability. *Catabacter hongkongensis*, *Phocaeicola vulgatus* and *Bacteroides caccae* were reliably identified from the FAA cultures while one of the unique colonies could not be reliably identified with MALDI-TOF MS. As the MALDI-TOF MS machine had not been updated to have the most recent taxonomical classifications, alternative classifications were added so that these results could be better compared to those from 16S V3V4 rRNA sequencing. Currently, *Catabacter hongkongensis* is called *Christensenella hongkongensis* and *Phocaeicola vulgatus* is *Bacteroides vulgatus.* Other classification updates were not found.

Table 8. Taxonomical classification results by MALDI-TOF MS from all of the differing bacterial colonies of the bacterial cultures of Donor 2's stool transplant.

Pure cultures were done from each of these colonies before MALDI-TOF MS measurement. The faeces were frozen for three years at -80°C before culturing. For some classifications, newer taxonomical names were added to parenthesis to allow for comparison with the sequencing results. FAA: Fastidious Anaerobe Agar plate, ORI: CHROMagar orientation agar plate.

Original Culture	Taxonomy	MALDI-TOF MS score
Lactose 1:100 1µl	*Escherichia coli*	2.35
Lactose 1:100 1µl	*Escherichia coli*	2.31
ORI 10µl	*Escherichia coli*	2.32
ORI 10µl	*Escherichia coli*	2.26
Blood 10µl	*Escherichia coli*	2.26
Blood 1:100 50µl	*Escherichia coli*	2.26
Blood 1:100 1µl	*Escherichia coli*	2.37
ORI 1:100 10µl	*Bacillus amyloliquefaciens*	2.00
FAA 1:100 10µl	*Catabacter hongkongensis (Christensenella hongkongensis)*	2.05
FAA 1:100 10µl	*Phocaeicola vulgatus* (*Bacteroides vulgatus*)	2.08
FAA 1:100 10µl	*Bacteroides caccae*	2.32
FAA 1:100 10µl	No reliable identification	< 1.5

The bacteria from the FAA and lactose undiluted 10µl agar plates were also collected separately to milk glycerol solutions, frozen at -80°C and sequenced with 16S V3V4 rRNA sequencing to see which bacteria could be identified from the cultures based on the bioinformatic analysis. The taxonomic profiles of the 20 most abundant OTUs in the bacterial cultures and their corresponding donation's samples according to SILVA OTU clustering are shown in Figure 12.

Of the MALDI-TOF MS-identified bacteria, *Escherichia coli*, *Christensenella* and *Bacteroides vulgatus* were also identified by SILVA OTU clustering from the 16S V3V4 rRNA sequenced bacterial culture samples and Donation 2.1 samples. However, *Christensenellaceae* had a low abundance in the bacterial culture samples (0.04%) and faecal samples (1.1-1.9%) even though it was identified with MALDI-TOF MS. The highest abundance of 1.9% was in the stool transplant sample from which the cultures were also made. Meanwhile, *Bacteroides caccae* and *Bacillus amyloliquefaciens* were not identified on a species level but might have been identified on a lower taxonomical level as a few *Bacillus* and many *Bacteroides* were identified from the samples. Their existence was supported by the fact that shotgun sequencing and taxonomic profiling were able to identify *Bacteroides caccae* from Donation 2.1's samples.

To observe the effect of freezing on the viability of the bacteria, the bacterial compositions of the cultures and faecal samples were compared. In this comparison, it was observed that *Bifidobacterium*, *Bacteroides, Alistipes putredinis, Parabacteroides merdae, Phascolarctobacterium faecium* and *Escherichia-Shigella* were viable even after being frozen for three years at -80°C. Other abundant bacteria from the faecal samples were not as abundant in the cultures. From Figure 12 it was also observed that the bacteria of the anaerobic FAA agar plate resembled the original gut microbiota composition more than the bacteria from the aerobic lactose agar plate.

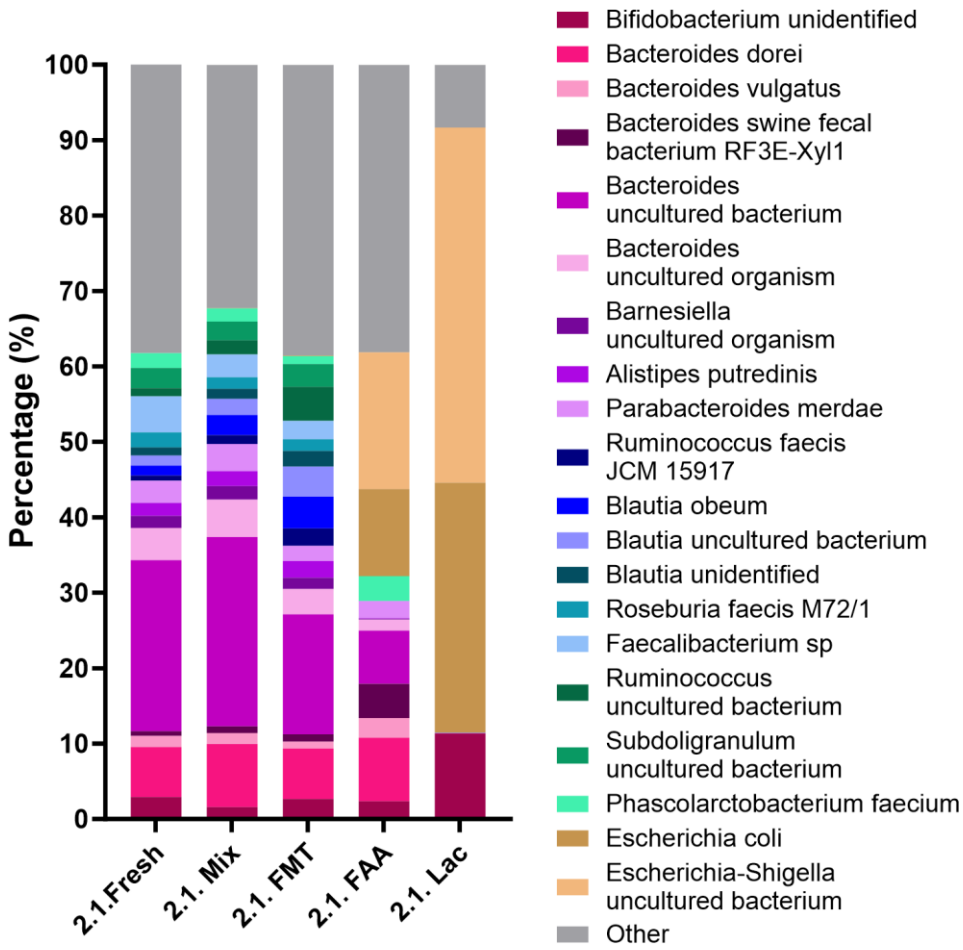


Figure 12. Bar chart of the 20 most abundant OTUs of the 16S V3V4 rRNA sequenced and SILVA OTU clustered bacteria cultured on Fastidious Anaerobe Agar (FAA) and lactose agar (Lac) plates (10µl undiluted) and the corresponding donation's (2.1) faecal samples. The OTUs are named on genus and species level, and they are in order based on their taxonomical relatedness. Fresh: fresh faecal sample, Mix: faecal mixture sample, FMT: stool transplant sample. Created in GraphPad Prism.

3 Discussion

This research aimed to determine how gut microbes are affected by being frozen at -80°C for up to three years in order to further validate and improve the stool transplant protocols used at the Turku Clinical Microbiome Bank. Additional goals were to see whether and due to which factors the gut microbiotas of healthy donors differ from each other, whether thawing overnight in a fridge differs from thawing at a 37°C water bath and whether parallel samples from the same donation differ from each other. This dataset was also used to evaluate the differences between 16S V3V4 rRNA and shotgun sequencing, Greengenes and SILVA reference databases, OTU clustering and ASV assigning and alpha and beta diversity methods.

3.1 The effects of freezing

No statistically significant differences in alpha or beta diversities between the original microbe compositions and the microbe compositions after freezing for up to three years were discovered. This means, first of all, that the glycerol and NaCl solutions added to the faecal sample during FMT processing did not statistically significantly affect the microbe composition of the samples and therefore the used protocol does not affect the quality of the stool transplants. This is in accordance with all the other studies proving that processing of the faeces with a cryoprotectant and NaCl solution does not significantly affect the microbe composition of the faeces or the effectiveness of FMT (Quraishi et al., 2017; Kao et al., 2017; Ianiro et al., 2018; Kim and Gluck, 2019; Cammarota et al., 2019).

Secondly, this also means that the species richness, evenness and composition of the gut microbes stayed unchanged even after being frozen at -80°C for three years. So, even after three years of frozen storage, the stool transplants should be usable and effective. This is an interesting finding, as it could lead to using stool transplants that have been frozen for over two years thus lowering the need of FMT providers to have multiple donors on hand. However, as the transplants were not given to patients after being frozen for three years but prior to that, as the currently recommended limit is two years of frozen storage (Cammarota et al., 2019), the in-patient effectiveness of the samples is unknown. As there are currently no other studies with over two years of frozen storage that could contradict these results, it is possible that the microbe composition does not significantly change even after three years of frozen storage. However, further research should be performed to confirm this, and the in-patient effectiveness of transplants frozen for over two years and to study at which point the gut microbes do change statistically significantly.

Thirdly, the stool transplant samples which all went through two freeze-thaw cycles differed more from the fresh faecal samples and faecal mixture samples than the two differed from each other although this difference was not statistically significant. This shows that an increase in the number of freeze-thaw cycles and/or a decrease in the concentration of the cryoprotectant do affect the gut microbes to some extent. However, interestingly the stool transplant samples had slightly higher alpha diversity values than the other samples in general instead of the expected lower values due to increased stress caused by the temperature changes and reduced glycerol concentration. As the alpha diversity values were also higher with the species richness methods, Total number and Chao 1 bias-corrected, the increase could not have been caused by a loss of bacterial species. Therefore, it is probable that the additional freeze-thaw cycle lowered the overall abundances of the species thus increasing the evenness of the sample without decreasing the number of species. This would be in accordance with the previously shown correlation between the number of freeze-thaw cycles and a decreased bacterial count (Packer et al., 1965). Additionally, it is also possible that during the diluting and filtering of the faecal mixture the stool transplant was exposed to contamination which would explain the slight increase in the species counts of these samples. Furthermore, it is also possible that the bacteria started to replicate during thawing thus increasing their numbers and evenness.

The differential abundance analyses showed that there were some bacteria which were differentially abundant in the fresh and faecal mixture samples. The differential abundance was checked on genus level so that closely related species with conflicting changes in abundance countered each other out. This gave rise to bacteria that generally increased or decreased due to being frozen at -80°C. Based on the differential abundance results, some *Actinobacteriota*, *Bacteroidota*, *Firmicutes* and *Verrumicrobiota* phyla all have some bacteria that tend to survive well in low temperatures over long periods of time while some *Cyanobacteria*, *Firmicutes* and *Proteobacteria* seem to be more sensitive to the cold. However, *Porphyromonas* was the only genus that was statistically significantly increased in abundance according to both sequencing methods so the generalizability of these changes might be low. Additionally, it is possible that the composition of the gut microbiota affects the sensitivity of each species to the cold resulting in contradictory changes in their abundances in different donors thus lowering the number of statistically significant differences. All in all, not many bacteria were statistically significantly differentially abundant and therefore most bacteria were not significantly or consistently affected by being frozen at -80°C.

The viability of the bacteria after being frozen for three years was checked by culturing the stool transplant solution of donation 2.1 on aerobic and anaerobic agar plates. The bacteria that grew on the plates were then classified with MALDI-TOF MS and sequenced with 16S V3V4 sequencing separately from the other samples. However, not all bacteria thrive in the culture conditions resulting in biased growth. Therefore, the viability of all of the bacteria cannot be reliably proven with bacterial cultures (Wensel et al., 2022). Furthermore, as the taxonomical classifications of MALDI-TOF MS and 16S V3V4 rRNA sequencing differed somewhat, it is difficult to determine which bacteria were viable. There are contradictory results on which method performs the best (Chen et al., 2020; Surányi et al., 2023), but at least the bacteria identified by both methods should have been viable in the samples.

Based on the results of 16S V3V4 rRNA sequencing and the fact that bacteria did grow on the agar plates, it is probable that most of the bacteria in the faecal samples were viable. The most abundant bacterial genera in the bacterial cultures according to 16S V3V4 rRNA sequencing were *Escherichia-Shigella* (51.1%), *Bacteroides* (13.8%), *Parabacteroides* (2.3%) and *Alistipes* (1.7%). These were all also abundant in the faecal samples although *Escherichia-Shigella* was not as abundant in the samples as it was in the cultures. The abundance of *Escherichia-Shigella* can be presumed to be high due to the culturable nature of these bacteria and to the aerobic plate accounting for half of the reads even though not many other bacterial strains of the gut can survive in aerobic conditions. Even though generally the bacterial cultures were similar to the faecal samples of donation 2.1, it is still possible that some of the bacteria detected in the faecal samples by sequencing were unviable and were not degraded before DNA extraction. In summary, it is probable that most of the sequenced bacteria were viable even though reliable confirmation of this is difficult to acquire due to the weaknesses of bacterial cultures.

3.2 Donor and donation differences

The alpha and beta diversities of the samples showed that different donors can have different species richness and evenness of gut microbes as well as a unique gut microbiota composition. These differences between donors' gut microbiotas are probably due to the individual differences between diet and lifestyle as these have been shown to affect the gut microbiota composition previously (Bressa et al., 2017; David et al., 2014; Fontana et al., 2019). Donors 1 and 2 differed the most from the other donors based on their gut microbiota profile while the other donors had more similar compositions with each other. However, each donor's samples still clustered closely together in the 2D PCoA scatterplots showing that the general gut microbiota composition stays

relatively unchanged even over long periods of time although changes in the relative abundances of species do occur as was seen from the alpha diversity results.

An exception to the close clustering was Donor 7's donations which were further apart from each other than some donors. This difference was seen in both alpha and beta diversities with alpha diversity showing a decrease in the number of bacteria in the gut. Additionally, the antibiotic resistance gene analysis also showed a major change between the donations with donation 7.3 having a lot more diverse antibiotic resistance genes than donations 7.1 or 7.2. Based on this information, it is probable that Donor 7 was ill and received antibiotic treatment in between these donations. Therefore, the changes in the gut microbiota of this donor are probably due to antibiotic treatment rather than natural changes due to dietary or lifestyle changes.

While each donor had their individual gut microbiota, statistically significant differences between the alpha diversities of the donations of a singular donor were observed showing that changes in the abundances of species do occur over time even though the microbiota composition stays relatively unchanged. This is probably due to changes in lifestyle, diet or health which can all change abruptly or slowly over time. This notion was supported by the fact, that faeces donated within a week of each other could be very similar (Donor 1) or somewhat different (Donor 2). Also, the faeces donated further apart (3 months to 1.5 years) showed that there can be significant changes over time in the number and abundances of species (Donor 7 and Donation 2.3) while it is also possible for these changes to reverse themselves (Donation 2.4). As there were only a few donors with more than one donation, these results are only indicative of the changes that can occur between donations.

No statistically significant differences between parallel samples of a donation were observed showing that the bacteria in the faeces are distributed quite evenly. However, this comparison only included two donations from one donor with a total of five sets of samples. Consequently, the result is only indicative of the possible differences between parallel samples.

3.3 Gender differences

In addition to the donors being different from each other, it was also discovered that females seem to have higher alpha diversity values than males in general suggesting that female faeces are more diverse and therefore possibly superior to male faeces in regard to FMT. Furthermore, the beta diversities also showed that females and males differed from each other statistically significantly with their microbe compositions suggesting that females and males also have differing gut microbe compositions. Similar results have been observed in also some larger studies (Lv et al., 2023; Kable

et al., 2022) showing that a more general pattern might exist although some contradictory results also exist (Fontana et al., 2019). The reason for female faeces being more diverse is probably found in their lifestyles and diets. Generally, females are more interested in their health and diet (Grzymisławska et al., 2020) causing them to eat more diversely which in turn can promote a more diverse gut microbiota.

The differential abundance analysis showed that in this data set females had higher abundances of some bacteria from the *Bacteroidota* and *Firmicutes* phyla while males had a higher abundance of some *Proteobacteria.* As *Proteobacteria* are overly abundant in CDI, having stool transplants more abundant in *Bacteroidota* and *Firmicutes* could increase the effectiveness of FMT. Therefore, this finding could lead to favouring female donors over male donors in the future. However, as the sample size was small, it remains to be seen whether these observations can be generalized.

3.4 Statistical analysis comparisons

3.4.1 Taxonomical classification methods

The 16S V3V4 rRNA sequenced reads were taxonomically classified with four differing methods: Greengenes OTU clustering, SILVA OTU clustering, ASV assigning in CLC Genomics Workbench and ASV assigning in Chipster. When the two reference databases, Greengenes and SILVA, were compared, SILVA was able to differentiate between similar sequences more effectively and classify more species-level classifications than Greengenes based on the OTU clustering analyses. SILVA OTU clustering also reached more similar results with shotgun sequencing and taxonomic profiling meaning that at least on the species level it is more accurate than Greengenes OTU clustering. Based on these factors, SILVA appears to be the better reference database of the two, especially if species-level classifications are wanted. However, Greengenes has been updated after the version used in this research, so the newer version might be able to give better results (McDonald et al., 2023).

The ASV assigning in the CLC Genomics Workbench had similar problems to the Greengenes OTU clustering as it also could not classify many of the reads up to the species level. ASV assigning in Chipster on the other hand was able to reach species-level classifications often. These classifications also correlated the best with those from shotgun sequencing and taxonomic profiling based on the ten most abundant species-level classifications.

As SILVA OTU clustering and ASV assigning in Chipster were compared to each other, it was noticed that both of these methods gave taxonomical classifications similar to each other and the shotgun-sequenced reads. ASV assigning in Chipster had five species level and one genus level match while SILVA OTU clustering had three species level and two genus level matches with the ten most abundant species-level taxonomical classifications of the shotgun sequencing. However, when the total amounts of taxonomical classifications of the faecal samples reaching species level were compared, SILVA OTU clustering had a 47.8% success rate while ASV assigning had a 31.9% success rate. Based on these factors, both methods worked well at classifying the reads accurately, but when species-level information is wanted SILVA OTU clustering appears to be slightly better than ASV assigning in Chipster.

In summary, SILVA OTU clustering was chosen as the taxonomical classification method for later analyses as it had the most species-level taxonomical classifications most of which could be matched to those from shotgun sequencing proving them to be accurate. ASV assigning in Chipster also performed well and could have therefore been used in further analysis instead of SILVA OTU clustering.

Currently, it seems that ASVs are preferred over OTUs because they are more comparable between studies and should provide more accurate results (Chiarello et al., 2022; Jeske and Gallert, 2022). However, neither ASVs nor OTUs are able to perfectly portray the true microbe composition of the samples and consequently, either can be used in data analysis as long as their strengths and weaknesses are accounted for. Furthermore, OTU clustering can be done with a 99% similarity cutoff making it more similar to ASV assigning without losing the benefit of including sequences with few mutations or sequencing errors in the analysis. In this study, a 97% similarity cutoff was used so that SILVA and Greengenes databases could be compared to each other.

3.4.2 Sequencing methods

Comparing the taxonomical classifications of 16S V3V4 rRNA sequencing and shotgun sequencing, it was observed that shotgun sequencing had a higher success rate with reaching species-level classifications, as was expected (Durazzi et al., 2021). Shotgun sequencing was able to classify 59.3% of taxonomical classifications of the faecal samples to the species level while SILVA OTU clustering of 16S V3V4 rRNA sequenced reads only reached the species level in 47.8% of OTUs. Therefore, the superiority of shotgun sequencing in this regard was clear.

The accuracy of these taxonomical classifications is harder to prove as the true microbe composition of the faecal samples is unknown. However, based on the positive controls of this study, 16S V3V4 rRNA sequencing performed better than shotgun sequencing as SILVA OTU clustering was able to identify correctly bacteria with lower abundances than taxonomic profiling, although at least one of these classifications was underestimated or misclassified on species level. Shotgun sequencing did not have noticeable misclassifications, but it was not able to identify as many of the bacteria in the positive controls as 16S V3V4 rRNA sequencing and SILVA OTU clustering. This could be due to the excessive filtering of the reads, the randomness of the sequences hindering identification or fragmentation of the sequences into too short pieces to be classified. In general, both methods were more accurate on the genus level than on the species level. In summary, as both sequencing methods had quite similar accuracies with the taxonomical classifications of the positive controls, but shotgun was able to reach species-level classifications more often and also made more in-depth analyses like antimicrobial resistance gene profiling possible, shotgun sequencing appears to be the better of the two sequencing methods.

3.4.3 Alpha diversity methods

Total number and Chao 1 bias-corrected methods both only consider species richness in their calculations which does not provide values that would be as descriptive of the true gut microbiota composition as taking both species richness and evenness into account (Haegeman et al., 2013). Therefore, they were not preferred over Shannon entropy or Simpson's index. However, the species richness methods are useful when trying to understand the causes behind certain changes seen with the other alpha diversity methods. For example, in this study, it would be hard to know whether the Shannon entropy of the stool transplant samples was increased due to diminished bacterial count causing higher evenness or due to higher species count causing increased richness. As these methods show how just the species richness develops, they can help with reaching the correct conclusions. In this study, the Total number and Chao 1 bias-corrected alpha diversity methods hardly differed from each other, so using only one of them should be enough to assess the changes in species richness.

Simpson's index and Shannon entropy both take species richness and evenness into account, but Shannon entropy gives more weight to the species richness while Simpson's index emphasizes species evenness. Both give more complex insights about the data which is good as the data itself is also complex. Straightforward comparison between the methods is difficult as both methods give alpha diversity values on completely different scales. Simpson's index values are always between 0

and 1 while Shannon entropy is not confined in the same way. This is why rather than comparing the values themselves, the size of the differences between groups of samples and the p-values associated with them were compared.

Based on these comparisons, it would seem that Shannon entropy gives often p-values between the species richness and Simpson's index methods. Additionally, as Shannon entropy allows for higher variation between samples due to not being bound to values between 0 and 1, it was visually easier to detect differences between the samples. Therefore, Shannon entropy was preferred over the other methods and used in the figures. However, all of the four methods showed the same trends in the data and thus resulted in the same conclusions meaning that any of these alpha diversity methods could have been used to describe the results. As the methods accentuate different aspects of the data, it is advisable to use a few of them to check that they give similar results instead of trusting only one of them.

3.4.4 Beta diversity methods

Both beta diversity methods gave very similar results to each other although Bray-Curtis was more sensitive to differences between groups as it gave higher pseudo-f-statistic values when differences were detected while Jaccard gave slightly higher p-values when differences were not detected. Essentially, either one of these methods could be used to analyse this type of data, but here Bray-Curtis was preferred and chosen for figures due to its slightly higher sensitivity to differences between groups. As with alpha diversity methods, it is useful to check the data with a couple of different beta diversity methods to get a better understanding of the range within which the differences vary.

Unfortunately, a phylogenetic method like Weighted UniFrac could not be included in this comparison as it requires specific sequences for each taxonomical classification which was not possible to provide due to the species-level aggregation of the data. However, even though phylogenetic methods take taxonomical relatedness better into account thus possibly lowering the significance of misclassifications, they are based on phylogenetic trees that have been constructed from the data. These trees are not always the most reliable sources of taxonomical relatedness which is why phylogenetic beta diversity measures can also be unreliable.

3.5 Strengths and weaknesses

This study has several strengths. First of all, the fact that three years of frozen storage was reached is a strength as there are no other studies that have studied the effect of freezing on gut microbes for this long. Also, the inclusion of multiple donors and several donations from individual donors allowed for donor and donation-wise comparisons. This increased understanding of how these factors can affect the microbe composition of stool transplants. Other strengths of this study are that two sequencing methods and multiple analysing methods were used which made in-depth comparisons of these methods possible.

There were also some weaknesses in this study. Firstly, the fact that the donations were not from the same donor or spaced evenly to fill the three-year collection time, made analysing the effect of freezing difficult. Secondly, the viability of the gut microbes was checked only from one sample and with bacterial cultures which is why the amount of dead bacteria in the samples is uncertain. Additionally, many of the factors that could be studied from this dataset had small sample sizes. For example, for gender comparisons, there were only four females and four males and for comparing the different thawing methods there were only two samples from one donor and donation. The low sample sizes make it difficult to generalize the results reliably, but they do give a prediction on what the generalization could be. As for the effectiveness of the transplants, no information on the effectiveness of the used transplants is available which is why the superiority of the donors could not be analysed.

3.6 Summary

In summary, this study did not find any statistically significant differences between the original microbe composition of the faeces and the microbe composition of the faecal mixture samples during three years of frozen storage at -80°C. Therefore, the primary outcome of this study was that as stool transplants are not statistically significantly affected by being frozen for up to three years, they could potentially be stored for three years or more without affecting the quality of FMT. More research is needed to ascertain what is the maximum time that gut microbes can be frozen for without affecting their viability or composition.

Additionally, no statistically significant differences caused by stool transplant processing were observed. However, donors did differ statistically significantly from each other showing that each individual has their own gut microbiota composition and therefore their samples cannot be reliably compared to each other. Even though donors differed from each other, they also had similarities

such as a 21% minimum abundance of *Bacteroides*. Also, based on the antimicrobial gene profiling they all had low antimicrobial resistance with tetracyclines being the most common antibiotic that the gut microbes had resistance to.

This study also showed a statistically significant difference between genders suggesting that females have more diverse gut microbiotas than males. Based on the differential abundance analysis, females have more bacterial species from the *Bacteroidota* and *Firmicutes* phyla while males have more *Proteobacteria*. This was an interesting finding, as it could lead to favouring female donors over male donors in treating CDI in the future. This is because FMT with high *Bacteroidota* and *Firmicutes* and low *Proteobacteria* compositions could be more effective as high abundance of *Proteobacteria* is associated with CDI. However, as the sample size was small, with four in each group, further research is needed to reliably prove these results.

When comparing statistical analysis methods, the SILVA reference database was found superior to Greengenes with 16S V3V4 rRNA sequences as it gave taxonomically higher and more accurate results. Additionally, OTU clustering and ASV assigning gave equally accurate results, but OTU clustering reached species-level taxonomical classifications more often and was therefore preferred over ASV assigning. From alpha diversity methods Shannon entropy was chosen as the most descriptive method while Bray-Curtis was preferred from the beta diversity methods, although it was almost identical to Jaccard. Even though these methods were preferred with this dataset, the use of a couple of different methods during data analysis is advisable.

Lastly, when 16S V3V4 rRNA and shotgun sequencing were compared, shotgun reached species level in taxonomical classification more often. Shotgun sequencing also allowed for more diverse analysis such as antimicrobial resistance gene profiling. Therefore, shotgun sequencing can be considered superior to 16S V3V4 sequencing when in-depth analyses are needed.

4 Materials and methods

4.1 FMT processing

The basis of this study was in faecal microbiota transplant processing (Figure 13). In this process, the faecal sample is first weighted and divided into ~30 g portions. Then, ~150 ml NaCl solution (0.09%) and 20 ml glycerol (85%) are added to create a faecal mixture solution. After the faecal mixture has been manually mixed it is frozen at -80°C until it is needed for faecal transplantation. On the day of faecal transplantation, the faecal mixture is thawed, diluted with ~130 ml of NaCl solution (0.09%) and filtered to get the finished stool transplant.

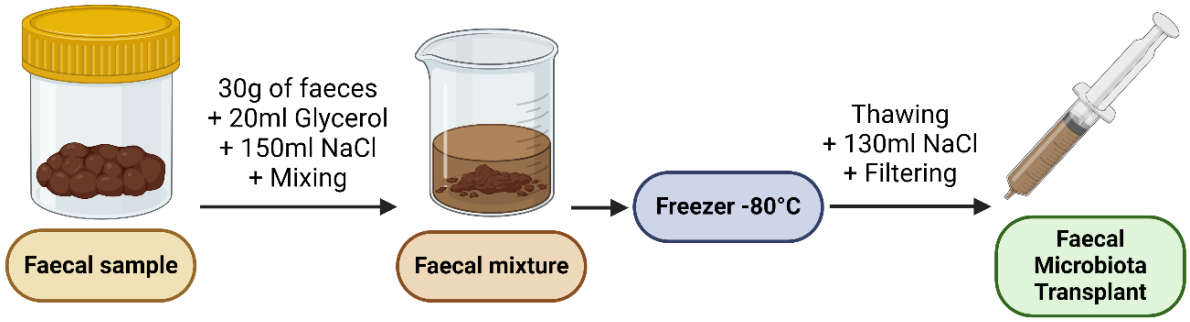


Figure 13. The faecal microbiota transplant processing protocol.

4.2 Sample collection

Faecal samples were collected from eight donors in total and processed into stool transplants during a three-year period. During the transplant processing, two to six ~2 ml samples were collected from the fresh faecal sample, faecal mixture and stool transplant. The samples collected from the fresh faecal material were put in OMNIgene·GUT solution (DNA Genotek, Ottawa, ON, Canada) to preserve the original gut microbiota composition and kept at RT for three days to normalise the time in the OMNIgene solution before freezing. In total, 16 sets of samples were collected, of which 13 were from different donations (Table 9). All samples were frozen at -80°C until DNA extraction.

Table 9. Information about the collected samples including donor numbers, donation numbers, time of donation (month/year) and the number of samples included in this study from each donation.

[1] Parallel sets of samples from the same donation, [2] The additional sample was used for bacterial cultures, [3] Parallel faecal transplant samples from the same donation, one with warm water thawing and one with overnight fridge thawing, [4] The fresh faecal sample was taken from donation 7.2 and other samples were taken from donation 7.1.

Donor	Donation	Donation time	Number of samples
1	1.1	08/2020	5 [1]
	1.2	08/2020	7 [1]
2	2.1	12/2020	4 [2]
	2.2	01/2021	4 [3]
	2.3	06/2022	3
	2.4	10/2022	3
3	3.1	02/2021	3
4	4.1	04/2021	3
5	5.1	10/2021	3
6	6.1	02/2022	3
7	7.1, 7.2	03/2022	3 [4]
	7.3	06/2022	3
8	8.1	07/2023	3

4.3 Bacterial culturing

Bacterial cultures were done from one donor's stool transplant sample to lactose, blood (5% sheep blood), CHROMagar orientation and FAA agar plates to confirm the viability of the bacteria after being frozen for three years. Four cultures from two concentrations and three volumes of the stool transplant were done on each agar plate type. The used volumes were 10µl of the undiluted stool transplant solution and 50µl, 10µl and 1µl of the stool transplant solution diluted to 1:100 with NaCl solution (0.09%). (Appendix 3)

The aerobic plates were incubated at 37°C for 24h and the anaerobic FAA plates were incubated at 37°C in anaerobic conditions (Oxoid AnaeroGen 2.5L Sachet - Thermo Scientific, Japan) for 96h. Pure cultures were done from all differing bacterial colonies and incubated for 24h at 37°C and 5% CO_2 or in anaerobic conditions. MALDI-TOF MS (Microflex LT, Bruker Daltonics, Billerica, MA, USA) was used to identify the bacterial strains of the colonies. All colonies from FAA 10µl and lactose 10µl cultures were put separately into milk glycerol and frozen at -80°C.

4.4 DNA extraction

The DNA extraction of the faecal samples was performed with Chemagic DNA Stool Kit special (PerkinElmer, Baesweiler, Germany) and Chemagic 360 instrument (PerkinElmer, Turku, Finland). First, 200µl of the samples was mixed with 800µl of lysis buffer and lysed with a PowerBead Pro Plate (Qiagen, Hilden, Germany) in TissueLyser II (Qiagen, Hilden, Germany) at 15Hz for 10 min. Then the bead plate was centrifuged for 6 min at 4500g after which 800µl of the lysed solutions were transferred to a high-well plate and 15µl of proteinase K was added. The plate was then incubated at 70°C for 10 min and at 95°C for 5 min before DNA extraction with the Chemagic 360.

The DNA of the frozen cultures was extracted similarly to the faecal samples, except that MN Bead Tubes Type B (MACHEREY-NAGEL, Düren, Germany) were used instead of the PowerBead Pro Plate. Additionally, 400µl of the milk glycerol colony mixture was mixed with 600µl of lysis buffer before lysing at 1200rpm for 8 min, and 600µl of the lysed solution was mixed with 200µl of lysis buffer and proteinase K before the incubation and DNA extraction.

ZymoBIOMICS Gut Microbiome Standard (Zymo Research, Irvine, CA, USA) was used as the positive control, while lysis buffer, glycerol (10%) and OMNIgene·GUT solution were used as negative controls. Additionally, OMNIgene Liquefaction reagent (DNA Genotek, Ottawa, ON, Canada) was added to all fresh faecal samples and Donor 8's faecal mixture sample to increase the fluidity of these samples. After the extraction, the DNA concentrations were measured with Qubit 1xdsDNA HS Assay Kit (Invitrogen, Eugene, OR, USA) after which the extracted DNA was divided into two 100µl portions, one for each sequencing method, and frozen at -80°C.

4.5 16S V3V4 rRNA sequencing

4.5.1 Amplicon PCR

The DNA of the faecal samples and ZymoBIOMICS Gut Microbiome Standard were diluted into 2.5 ng/µl with UltraPure™ Distilled Water DNase/RNase Free (Invitrogen, Paisley, UK) according to the results of the previous Qubit measurements. ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, Irvine, CA, USA) diluted into 2.5 ng/µl with UltraPure Distilled Water was used as the positive control for the 16S V3V4 rRNA Sequencing. The negative controls: lysis buffer, glycerol (10%), OMNIgene solution and UltraPure Distilled Water (negative control for 16S V3V4 rRNA sequencing), were included as undiluted.

The 16S V3V4 hypervariable rRNA regions were multiplied with a master mix, that was comprised of 16.5µl 2xKAPA Hifi hotstart ready mix Roche, 1µl of V3V4 forward and reverse primers (6.6µM, metabion international AG, Planegg, Germany) and 7.9µl of UltraPure Distilled Water per sample. The master mix was put in 8 wells after which 6.6µl of each diluted DNA template (2.5 ng/µl) was put into its own well containing the master mix so that the total volume was 33µl/well. The strips were then put into a T100 Thermal Cycler (BIO-RAD, Singapore) for amplification of the V3V4 hypervariable rRNA regions of each sample. The used thermal program was 95°C for 3 min, [95°C for 30s, 55°C for 30s, 72°C for 30s] x25, 72°C for 5 min, 4°C for ∞. The successfulness of the amplification was confirmed with gel electrophoresis.

In the gel electrophoresis, 8µl of each amplified sample was mixed with 2µl of 6x DNA Loading Dye (Thermo Scientific, Vilnius, Lithuania), and 8µl of this dyed DNA mixture was put on a 1.5% TAE agarose gel (Standard Agarose -Type LE, BioNordika, Helsinki, Finland) with MIDORI Green Advance DNA Stain (Nippon Genetics, Düren, Germany). 5µl of GeneRuler 100bp DNA Ladder (Thermo Scientific, Vilnius, Lithuania) was used as an indicator of DNA fragment size on each side of the samples. The gel was run at 120V in 1xTAE solution for one hour with PowerPac Basic (BIO-RAD, Singapore) and imaged with Universal Hood II (BIO-RAD, USA).

The amplified V3V4 hypervariable rRNA regions were purified with 20µl of AMPure XP Beads (Beckman Coulter Diagnostics, Brea, CA, USA). After the beads were added to the samples, the mixture was incubated at RT for 5 min while manually turning the strip. After a 2 min additional incubation at RT on a magnetic plate, the supernatant was removed, and the beads were washed twice with 200µl of fresh 80% ethanol with a 30s incubation in between. Then the beads were left to dry until the surface of the bead pellet was mat (~ 6 min) and resuspended in 52.5µl of UltraPure Distilled Water. This was followed with a 2 min incubation at RT and a 2 min incubation at RT on a magnetic plate. After the incubations 48µl of the supernatants were transferred into a new strip. The purified sample strips were stored at -20°C.

4.5.2 Index PCR

To amplify the indexed sequences, a new master mix with 10µl of UltraPure Distilled Water and 25µl of 2xKAPA Hifi hotstart ready mix per sample was done. On top of this, 10µl of IDT for Illumina DNA/RNA UD Indexes Set A or B (Illumina, San Diego, CA, USA) were added to each strip well. Lastly, 5µl of the purified amplicon PCR template was added to the mixture (total volume 50µl/well). The strip was then put into a T100 Thermal Cycler (BIO-RAD, Singapore) for amplification of the indexed V3V4 hypervariable rRNA regions of each sample. The used thermal

program was 95°C for 3 min, [95°C for 30s, 55°C for 30s, 72°C for 30s] x8 (or x9 with some samples), 72°C for 5 min, 4°C for ∞.

The amplified V3V4 hypervariable rRNA regions with indexes were purified with 56µl of AMPure XP Beads (Beckman Coulter Diagnostics, Brea, CA, USA). After the beads were added to the samples, the mixture was incubated at RT for 5 min while manually turning the strip. After a 2 min additional incubation at RT on a magnetic plate, the supernatant was removed, and the beads were washed twice with 200µl of fresh 80% ethanol with a 30s incubation in between. Then the beads were left to dry until the surface of the bead pellet was mat (~ 6 min) and resuspended in 27.5µl of UltraPure Distilled Water. This was followed with a 2 min incubation at RT and a 2 min incubation at RT on a magnetic plate. After the incubations 25µl of the supernatants were transferred into a new strip.

The DNA concentration of the purified V3V4 hypervariable rRNA regions with indexes was measured with Qubit dsDNA BR Assay Kit (Invitrogen, Eugene, OR, USA), Qubit assay tubes (Invitrogen, Eugene, OR, USA) and Qubit 4 fluorometer (Invitrogen, Eugene, OR, USA), to ensure the successfulness of the protocol and to calculate the pooling volumes. 2µl of the purified samples were used per measurement and each sample was measured twice parallelly. The purified sample strips were stored at -20°C until pooling and sequencing.

4.5.3 Sequencing

The purified V3V4 hypervariable rRNA regions with indexes were diluted into 4nM with UltraPure Distilled Water according to the Qubit concentration measurements and expected fragment size. The samples were then pooled together by combining 5µl of each sample in an Eppendorf.

Next, 2µl of the PhiX Control v3 (Illumina, San Diego, CA, USA) was diluted in 3µl of UltraPure Distilled Water. 5µl of 0.2 M NaOH was added to the dilution and incubated at RT for 5 min. Then, 990µl of Hyb Buffer (Illumina, San Diego, CA, USA) was added. This solution was then diluted to 8 pM by mixing 20µl of it with 30µl of Hyb Buffer.

The combined sample solution (library) was denatured by mixing 5µl of the 4 nM pooled solution with 5µl of 0.2 M NaOH. The mixture was incubated at RT for 5 min after which 990µl of Hyb Buffer was added. This denatured library was then further diluted by mixing 114µl of it with 456µl of Hyb Buffer.

The denatured and diluted PhiX control was then combined with the denatured and diluted library by adding 30µl of the 8 pM PhiX control to the library. The mixture was then incubated at 95°C for 2 min and on ice for 5 min. After the incubations, the solution (600µl) was loaded into the MiSeq® Reagent Kit v3 600 Cycles PE (Illumina, San Diego, CA, USA) reagent cartridge. Then the flow cell was washed with fresh MQ-water and 80% ethanol after which the library was sequenced with MiSeq™ (Illumina, San Diego, CA, USA).

4.6 Shotgun sequencing

The DNA of the faecal samples and ZymoBIOMICS Gut Microbiome Standard were diluted into 0.2 ng/µl with UltraPure Distilled Water, DNase/RNase Free (Invitrogen, Paisley, UK) according to the Qubit measurements. ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, Irvine, CA, USA) diluted into 0.2 ng/µl with UltraPure Distilled Water was used as the positive control for the shotgun sequencing. The negative control from DNA extraction, lysis buffer, was included as undiluted.

4.6.1 Tagmentation

Nextera XT Library Prep Kit (Illumina, San Diego, CA, USA) was used to prepare the Shotgun library. First, 10µl of Tagment DNA Buffer, 5µl of Amplicon Tagment Mix and 5µl of DNA (0.2ng/µl) were put into 8 well strips. The DNA was then tagmented in the Thermal Cycler with a "55°C for 5 min and 10°C for ∞" -program. After the sample temperature reached 10°C, 5µl of Neutralize Tagment Buffer was added to each well. The strip was then incubated at RT for 5min.

4.6.2 Amplification

10µl of IDT for Illumina DNA/RNA UD Indexes and 15µl of Nextera PCR Master Mix were added to each strip well. Then the indexed DNA fragments were amplified in the Thermal cycler with a "72°C for 3 min, 95°C for 30s [95°C for 10s, 55°C for 30s, 72°C for 30s] x12, 72°C for 5 min, 10°C for ∞" -program.

4.6.3 Purification

The amplified DNA was purified with 25µl of AMPure XP Beads (Beckman Coulter Diagnostics, Brea, CA, USA). After the beads were added to the samples, the mixture was incubated in a shaker at 1800 rpm for 2 min and subsequently at RT for 5 min. After a 2 min additional incubation at RT on a magnetic plate, the supernatant was removed, and the beads were washed twice with 200µl of

fresh 80% ethanol with a 30s incubation in between. Then the beads were left to dry for 5 min after which they were resuspended in 52.5µl of Resuspension Buffer. This was followed with a 2 min incubation at RT and a 2 min incubation at RT on a magnetic plate. After the incubations 20µl of the supernatants were transferred into two new strips. One strip was used for concentration and bioanalyzer measurements, while the other was kept at -20°C until sequencing to minimize the freeze-thaw cycles.

4.6.4 Quantitation

The DNA concentration of the shotgun sequenced samples was measured with Qubit 1xdsDNA HS Assay Kit (Invitrogen, Eugene, OR, USA), Qubit assay tubes (Invitrogen, Eugene, OR, USA) and Qubit 4 fluorometer (Invitrogen) to ensure the successfulness of the protocol and to calculate the pooling volumes. 2µl of the purified samples were used per measurement and each sample was measured twice parallelly.

4.6.5 Fragment size estimation

To calculate the pooling volumes, the average fragment size of each sample was measured with 2100 Bioanalyzer (Agilent Technologies), High Sensitivity DNA Chips (Agilent Technologies, Waldbronn, Germany) and High Sensitivity DNA Reagents (Agilent Technologies, Waldbronn, Germany). First, 9µl of gel-dye mixture was pipetted into one of the four gel wells. Then, the pistol of the loading station was pushed down from 1ml and incubated in the down-position for 1 min. Then 9µl of the gel-dye mixture was added to the other three gel wells. 5µl of the marker was put into each of the 11 sample wells and the ladder well. Then, 1µl of the ladder was put into the ladder well and 1µl of each sample was put into their own wells. The chip was then shaken at 2400 rpm for 1 min after which it was placed in the Bioanalyzer, and the wells were analysed. The average fragment size was calculated based on a manually set area.

4.6.6 Sequencing

The purified shotgun samples were diluted into 2 nM with NextSeq 1000/2000 Resuspension Buffer with Tween 20 (Illumina, San Diego, CA, USA) according to the Qubit's concentration measurements and Bioanalyzer's average fragment size. The dilutions were made into DNA LoBind Tubes (Eppendorf SE, Hamburg, Germany). The samples were then pooled together into a library by combining 5µl of each sample dilution.

1.75µl of the PhiX Control v3 (Illumina, San Diego, CA, USA) was diluted in 98.3µl of NextSeq™ 1000/2000 Resuspension Buffer with Tween 20 (Illumina, San Diego, CA, USA). Then, 24µl of the pooled library was mixed with 1µl of the PhiX control dilution. 20µl of this solution was then put into the NextSeq™ 1000/2000 P2 Reagent Cartridge, 300 cycles (Illumina, San Diego, CA, USA) and sequenced with NextSeq™ 2000 (Illumina, San Diego, CA, USA).

4.7 Statistical analysis

CLC Genomics Workbench (Qiagen, version 23.0.2) and Chipster (CSC, R versions 4.1.1 and 4.2.0) were used to perform the analyses. In Chipster the "Read quality with MultiQC for many FASTQ files" -function was used to visualize read quality before analysis and after trimming the primers (Appendix 2). In CLC, read quality was assessed with the "QC for Sequencing Reads" -function. The indexes were trimmed automatically in Basespace (Illumina), so that process was not included in any of the pipelines. The general analysis pipelines of 16S V3V4 rRNA and shotgun sequencing are shown in Figures 14 and 15, respectively.

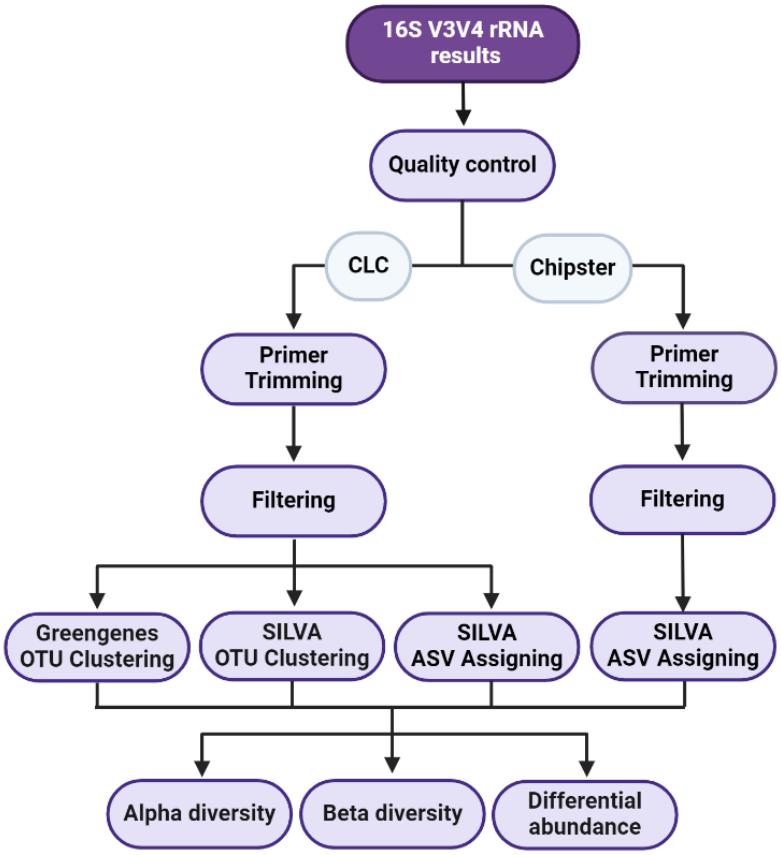


Figure 14. Analysis pipeline of the 16S V3V4 rRNA sequenced samples. Created in BioRender.

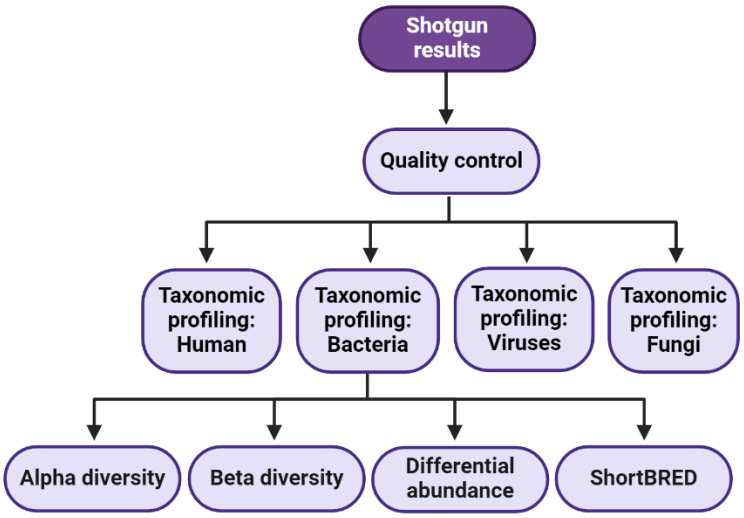


Figure 15. Analysis pipeline of the shotgun sequenced samples. Created in BioRender.

4.7.1 Taxonomical classification (16S V3V4 rRNA sequencing)

Four different pipelines were used to taxonomically classify the 16S V3V4 rRNA sequenced reads: OTU clustering with Greengenes reference database, OTU clustering with SILVA reference database, ASV assigning with SILVA reference database in CLC Genomics Workbench and ASV assigning with SILVA reference database in Chipster. The OTU clustering analyses were both performed in CLC Genomics Workbench.

In the taxonomical classification pipelines performed in CLC, the reads were trimmed based on quality scores (limit 0.05) and the number of ambiguous nucleotides (max 2). Then, the primers were removed based on the known primer sequences and automatic read-through. Reads shorter than 200 bases were removed, as these were deemed to be unsuccessfully sequenced reads based on the data quality analysis. After trimming, the reads were filtered so that all samples with less than 100 reads were discarded.

OTU clustering was performed separately with two reference databases, Greengenes 97% (v13_8) and SILVA SSU 99% (v138.1) (Table 10). In both clustering methods, creation of new OTUs was allowed, taxonomy similarity between reads under the same OTU was set to 80% and similarity to reference database was set to 97%.

Table 10. Names and sizes of the reference databases used with the analyses of 16S V3V4 rRNA sequenced samples according to CLC Genomics Workbench.

Reference Database	Size (sequences)
Greengenes 97% (v13_8)	99 322
SILVA SSU 99% (v138.1)	510 508

In ASV assigning in CLC, the ASVs were assigned to reads with the "Detect Amplicon Sequence Variants" -tool. The forward reads were trimmed to 250 bases and reverse reads to 200 bases based on the quality results. All chimaeras were removed. Then, the ASVs were assigned taxonomy based on the SILVA reference database. The minimum similarity between an ASV and the database reference sequence was set to 80%.

In Chipster, the primers were trimmed with the "Remove primers and adapters with Cutadapt" -function. Then the reads were trimmed and filtered with the "Filter and trim sequences with DADA2" -function so that the forward reads were trimmed to 250 bases and reverse reads to 200 bases similar to CLC. Additionally, reads with ambiguous bases or less than 200 bases were removed, and reads were truncated after a base quality of two. Next, the reads were handled with the "Sample Inference" -function after which the reads were made into contigs with the "Combine paired reads to Contigs with DADA2" -function. At least 12 bases overlap and 80% bootstrapping confidence were required. Then, the contigs were gathered into an ASV table and assigned to taxonomy with the "Make ASV table and remove Chimeras" and "Assign Taxonomy" -functions. SILVA v.138.1 reference database was used in the taxonomy assignment. To visualize and add metadata to the ASV-Taxonomy table a phyloseq object was made with the "Make a phyloseq object", "Merge phenodata to phyloseq object", and "Extract information from phyloseq object" -functions. Then the table was imported into CLC for better and more comparable visualization and for alpha and beta diversity analyses.

4.7.2 Taxonomical classification (Shotgun sequencing)

The shotgun sequenced reads were taxonomically classified with the "QC and taxonomic profiling" -pipeline. The reads were first trimmed based on their quality score (limit 0.05) and ambiguous nucleotides (max 2). Automatic read-through adapter trimming was enabled. Based on the quality analysis, reads shorter than 140 bases were removed.

Taxonomic profiling was performed in two steps. First, the bacterial reads were assigned taxonomy according to the Unified Human Gastrointestinal Genome (UHGG) (01/2022) reference database

and human reads were filtered as the host reads with the Homo Sapiens (GRCh38) reference database. Second, the unmapped reads that were neither bacteria nor human were assigned taxonomy according to the clustered Reference Viral DataBase (RVDB) (21.0, 06/2021) with Microbial Genome Database (09/2023) as the host (Table 11). The second taxonomy assignment was done to see whether the unmapped reads were from viruses and fungi or disregarded for some other reason.

Table 11. Names and sizes of the reference databases used with the analysis of shotgun-sequenced samples according to CLC Genomics Workbench.

Reference Database	Size (sequences)	Size (bp)
UHGG (01/2022)	679 691	11 175 027 434
Homo Sapiens (GRCh38)	25	3 088 286 401
RVDB (21.0, 06/2021)	805 112	2 141 575 027
Microbial Genome Database (09/2023)	1 052	2 595 817 375

4.7.3 Alpha and beta diversity

First, taxonomical classifications with low abundances were removed, so that only those that had more than 10 reads, were kept. With alpha diversity, the maximum depth was set to 200,000 so that the different methods could be compared to each other at the same rarefaction level. The alpha diversities were calculated with the Total number, Chao 1 bias-corrected, Simpson's index and Shannon entropy methods on species-aggregated data. The formulas used for the calculations were $Chao\ 1\ (bias-corrected) = D + \frac{f_1(f_1-1)}{2(f_2+1)}$, $Simpson's\ index\ (complemented) = 1 - \sum_{i=1}^{n} p_i^2$ and $Shannon\ entropy = \sum_{i=1}^{n} p_i log_2 p_i$, in which n was the number of features and p_i was the fraction of reads that belonged to feature i, D was the number of distinct features in one sample, f_1 was the number of features that only had one read and f_2 was the number of features that had two reads (CLC Microbial Genomics Module USER MANUAL User manual for CLC Microbial Genomics Module 24.0).

The beta diversities were measured with Bray-Curtis and Jaccard methods on species-aggregated data. The formulas used for these calculations were $Bray-Curtis = \frac{\sum_{i=1}^{n} |x_i^A - x_i^B|}{\sum_{i=1}^{n} (x_i^A - x_i^B)}$ and $Jaccard = 1 - \frac{\sum_{i=1}^{n} \min(x_i^A, x_i^B)}{\sum_{i=1}^{n} \max(x_i^A, x_i^B)}$ in which n was the number of OTUs or ASVs and x_i^A and x_i^B were the abundances of OTU/ASV i in samples A and B, respectively (CLC Microbial Genomics Module USER MANUAL User manual for CLC Microbial Genomics Module 24.0).

The differences between beta diversities of different sample groups were calculated with PERMANOVA. The examined sample groups were sample type, donor and gender. The sample type comparison was used to compare the effect of freezing and stool transplant processing on the microbe compositions of the faecal samples. Donor comparisons were done to examine how different each donor's gut microbiota composition was compared to the others. Gender comparison was used to see if the microbe compositions of females and males differed from each other. The beta diversities were also visualized with 2D principal coordinate scatterplots while alpha diversities were visualised with boxplots by grouping the alpha diversity results.

4.7.4 Differential abundance

In order to analyse the changes in specific bacteria due to frozen storage, differentially abundant genera were analysed with the "Differential Abundance Analysis" -tool in CLC Genomics Workbench after the taxonomical classifications were aggregated on a genus level. The effect of frozen storage on the abundance of bacterial genera was analysed by comparing the composition of the fresh faecal samples and faecal mixture samples as the first represents the original microbe composition and the second represents the microbe composition after being frozen. All samples were taken into the analysis so that general patterns could be recognised better. Additionally, the differential abundance of bacterial species due to gender was also calculated from all of the samples after species-level aggregation.

4.7.5 Antimicrobial resistance

ShortBRED was used to identify antimicrobial resistance genes from the shotgun sequenced and taxonomically profiled bacterial reads based on the QMI-AR Peptide Marker Database (08/2021). DIAMOND was used to identify the antibiotic resistance markers. The analysis was performed in the CLC Genomics Workbench with the "Find Resistance with ShortBRED" -tool.

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6 Abbreviations

%Q30	The percentage of bases with a quality score of 30 or higher
ASV	Amplicon Sequence Variant
CDI	*Clostridioides difficile* infection
FDR p-value	False discovery rate p-value
FMT	Faecal Microbiota Transplantation
MALDI-TOF MS	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
OTU	Operational Taxonomical Unit
PCoA	Principal Coordinates Analysis
PERMANOVA	Permutational Multivariate Analysis of Variance
ShortBRED	The Short, Better Representative Extract Dataset
UHGG	The Unified Human Gastrointestinal Genome reference database

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Appendices

Appendix 1 Sequencing quality

The qualities of the sequencings were checked with several methods. First, the quality metrics received from the sequencing were checked so that they were in accordance with the outcomes promised by the manufacturer. These quality metrics included the yield of the sequencing in base pairs and the %Q30. In 16S V3V4 rRNA sequencing the yield was 14.48 Gbp and the %Q30 was 75.63. In shotgun sequencing, the yield was 157.67 Gbp and the %Q30 was 88.10. (Table A1) All of these values except for the %Q30 of shotgun sequencing were within the manufacturer's expected ranges proving that both sequencings were successful. The %Q30 of shotgun sequencing was less than 2% under the expected 90% value, so it was still relatively high indicating that most of the reads in the sequencing were of good quality.

Table A1. Sequencing quality metrics of both 16S V3V4 rRNA and shotgun sequencing and their expected values according to the manufacturer of the sequencing kits.

%Q30: the percentage of bases with a quality score of 30 or higher

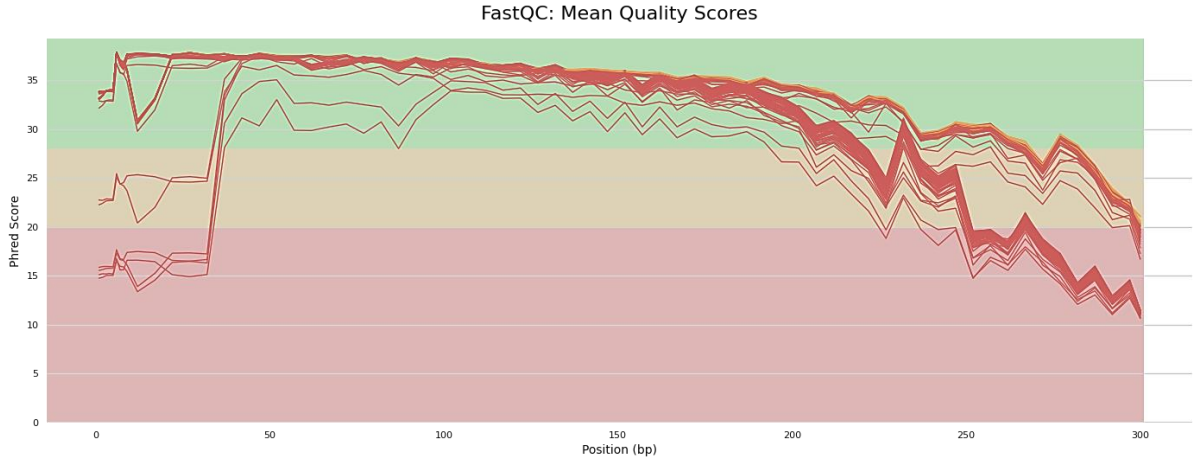
Sequencing quality	16S V3V4 rRNA sequencing		Shotgun sequencing	
	Actual value	Expected value	Actual value	Expected value
Yield (Gbp)	14.48	13.2 – 15	157.67	120
%Q30	75.63	> 70%	88.10	≥ 90%

Appendix 2 Read quality

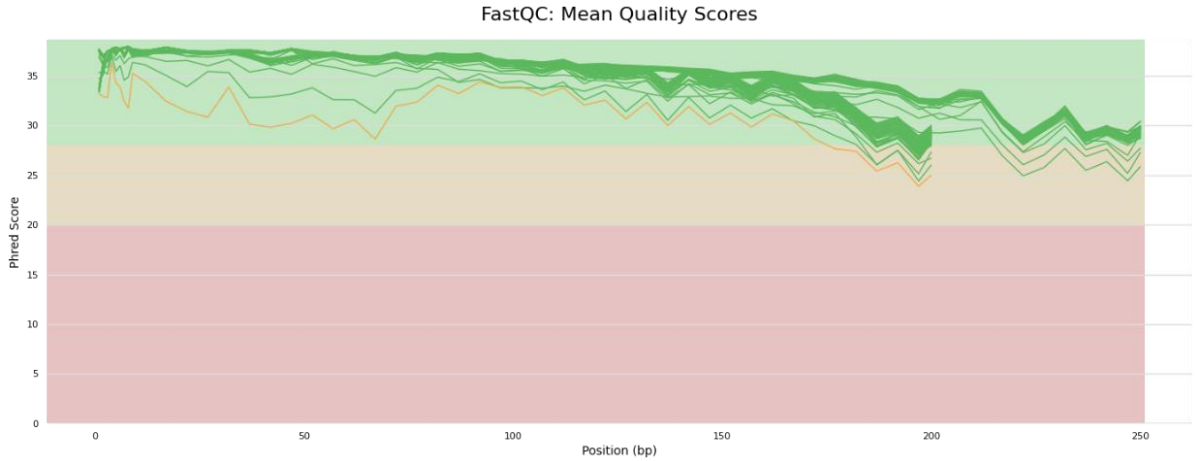
The quality of the reads was checked with MultiQC in Chipster. In 16S V3V4 rRNA sequencing most of the forward and reverse reads of the samples (81 sets in total) failed the quality test while 23 were given warnings (Figure A1A). None of the reads passed the quality test without problems. However, this test was done prior to any modifications, so the reads still had the primers attached to them and none of the overly short reads had been removed. After the primers were removed, short reads were filtered and low-quality ends were trimmed, 103 of the 104 forward and reverse reads of the samples passed the quality test while the reverse reads of one negative control still received a warning (Figure A1B).

In addition to the quality score, the length distributions of the reads were checked with MultiQC in Chipster to see how well the sequencing had worked and which length value should be used for filtering overly short reads. In 16S V3V4 rRNA sequencing most of the reads were 275-301 bases long (Figure A1C) while most of the few shorter reads were approximately 30-40 bases, 80-90 bases, 125-140 bases, 160 bases or 180 bases long. As most of the shorter reads were less than 200 bases long, that was set as the lowest allowed read length value for filtering.

A) Read quality after 16S V3V4 rRNA sequencing



B) Read quality after trimming and filtering



C) Length distribution after 16S V3V4 rRNA sequencing

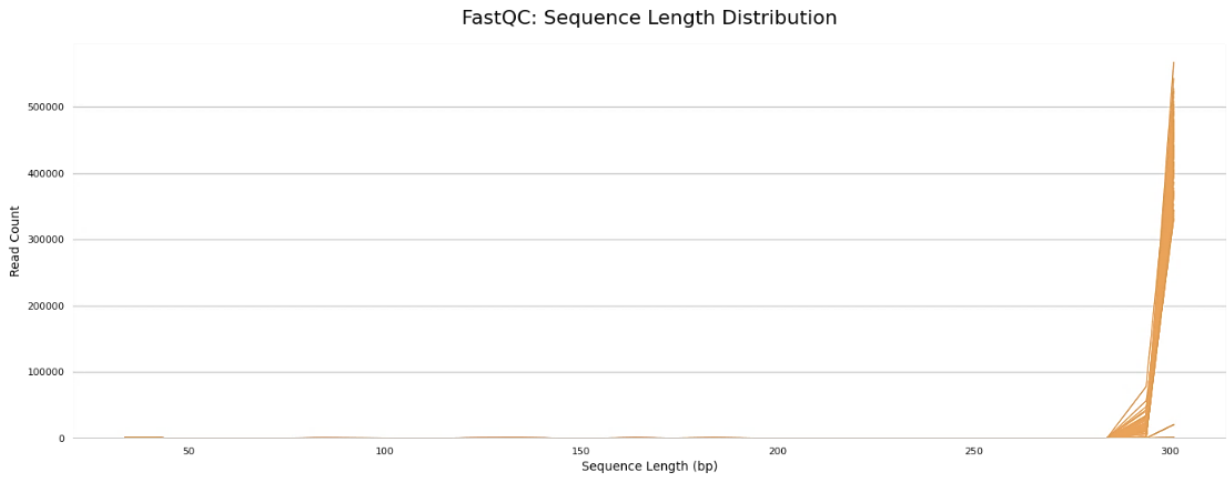
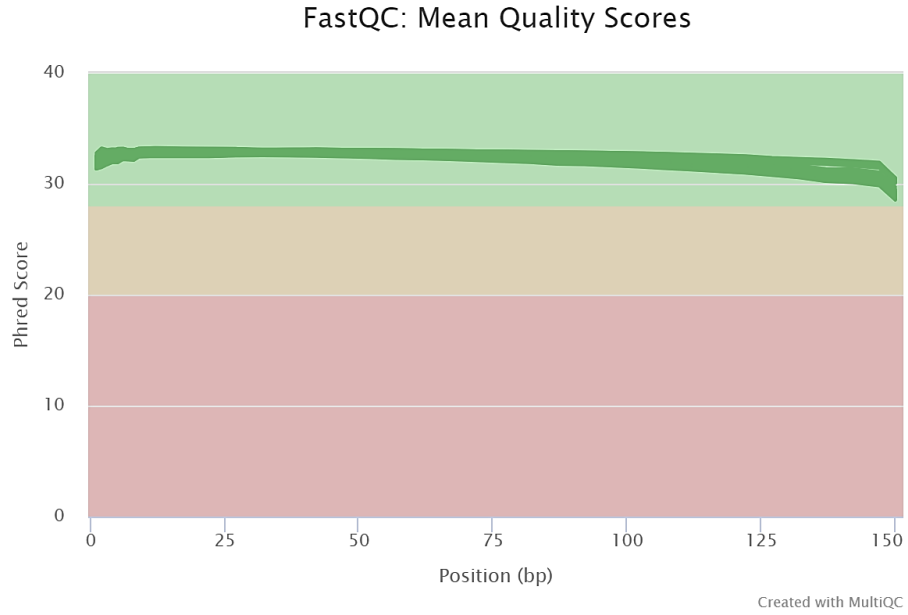


Figure A1. A) Mean quality scores of the 16S V3V4 rRNA sequenced reads of the samples before any improvements. 23 of the forward and reverse reads of samples received warnings while the rest (81) failed the quality test. B) Mean quality scores of the 16S V3V4 rRNA sequenced reads of the samples after trimming and filtering. 103 of the forward and reverse reads passed the quality test while one of the reverse reads of the negative control received a warning. C) The length distribution of the reads of the samples after 16S V3V4 rRNA sequencing. Most of the reads were 275-301 bases long although some shorter reads were under 200 bases. The analyses were performed with MultiQC in Chipster.

In shotgun sequencing all of the 98 forward and reverse reads of the samples passed the quality test before any modifications were made (Figure A2A). However, the read length distribution showed a lot of reads that were shorter than the expected 150 bases (Figure A2B). The reads were filtered to reduce the amount of faulty and short reads so that only reads longer than 140 bases were kept. As most of the reads were 140-150 bases long this did not affect the data significantly.

A) Read quality after shotgun sequencing



B) Length distribution after shotgun sequencing

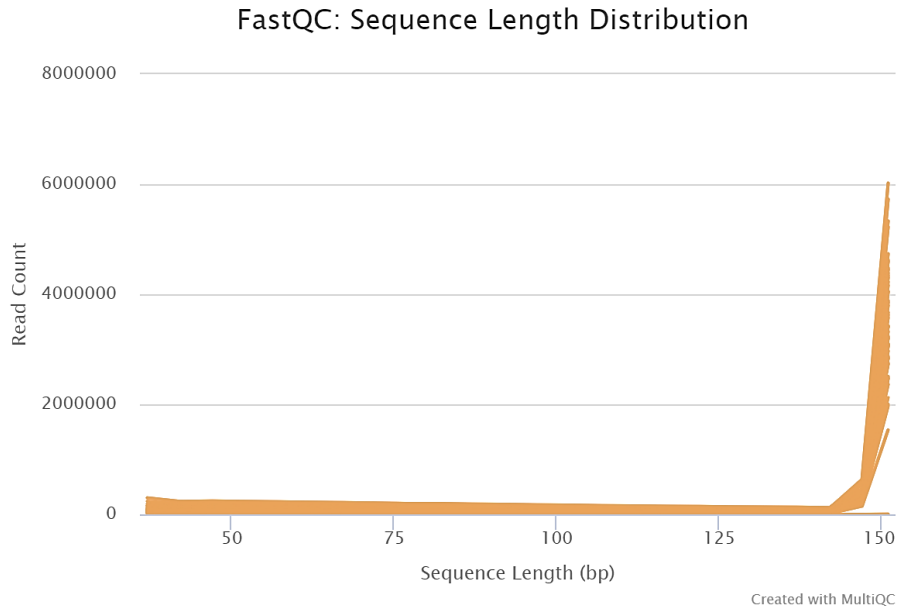


Figure A2. A) Mean quality scores of the shotgun sequenced reads of the samples after sequencing. All of the 98 reads passed the quality scoring without any modifications. B) Length distribution of the shotgun sequenced reads. Most of the reads were 140-150 bases long although many reads were under 140 bases. The analyses were done with MultiQC in Chipster.

Appendix 3 The bacterial cultures of Donation 2.1

The bacterial cultures made from the stool transplant sample of Donation 2.1 were photographed before pure cultures were done from the unique colonies (Figure A3). The aerobic cultures mostly had similar-looking colonies, although there was one blue colony in the 1:100 10µl CHROMagar orientation plate which clearly differed from the others. Meanwhile, the anaerobic FAA agar plate had more variable colonies.

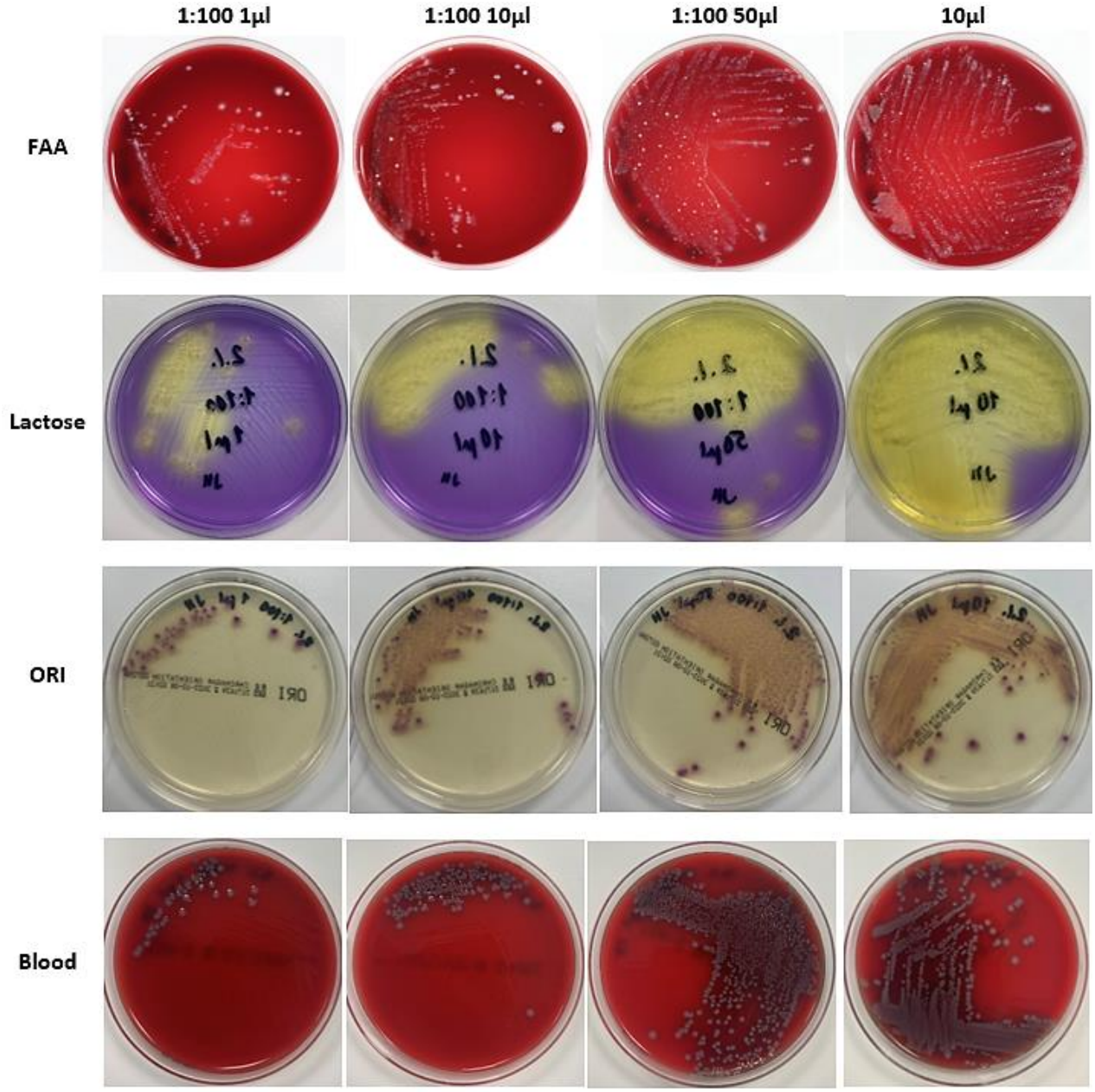


Figure A3. Images from all the bacterial cultures made from Donor 2's stool transplant. The faeces were frozen for three years at -80°C before culturing. FAA: Fastidious Anaerobe Agar plate, ORI: CHROMagar orientation plate.