Faculty of Health Sciences

Development of quantitative PCR method for *Escherichia coli* detection in human stool samples

Thomas Lied

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Supervisor: Veronika Kuchařová Pettersen, Associate Professor at Host-Microbe Interaction research group, Department for Medical Biology, UiT Arctic University of Norway

Preface

This project was conducted during the school year of 2023/2024 from the 15th of August 2023 until the 12th of June 2024. It was performed at the Host-Microbe Interaction (HMI) research group at the Department of Medical Biology at UiT the Arctic University of Norway. My Supervisor was Veronika K. Pettersen, Associate Professor at HMI, Department of Medical Biology, and my co-supervisor Wasifa Kabir

First of all, special thanks to my supervisor, Veronika Pettersen, who went way beyond expectations in supervising me and taking me on as a master student. Veronika gave invaluable guidance, support, and insightful feedback and was always available when I needed it the most throughout the course of this year. Thank you for your immense patience. I would also like to thank Wasifa Kabir for your technical support in the lab.

Thank you, Host Microbe Interaction, for letting me be part of this wonderful research group. I had so much fun getting to know you all, and I am forever grateful for everything I have learned while being part of this research group; I got an opportunity to learn useful skills way past the need for this thesis. Nobody was ever bothered by my stupid questions, took me in, and treated me as an equal, from learning to use a pipette to finish this thesis. Wish you all the best.

Finally, special thanks to my friends and family who have supported along the way.

Disclosure: I used Gramarly for improving my written English language in the whole thesis.

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Abstract

The gut microbiome is a complex community of microorganisms that reside in the gastrointestinal tract of mammals and other animals. This diverse ecosystem includes *Escherichia coli* (*E. coli*) bacteria, which are not just present, but are essential in maintaining health by aiding digestion, synthesising vitamins such as Vitamin B_{12} and K, and protecting against pathogens. *E. coli* are versatile bacteria including various strains, some of which are harmless commensals, while others can cause serious infections. In a healthy gut, *E. coli* contribute significantly to the microbiome's balance as a normal gut inhabitant. However, pathogenic strains can cause severe foodborne illnesses, such as diarrhoea, abdominal cramps, and, in extreme cases, kidney failure.

The aim of this thesis work was to develop and optimise a protocol for absolute quantification of *E. coli* from human stool. First, optimisation of DNA extraction from human faecal samples was performed, ensuring the purity and quantity of the DNA is optimal for the downstream application, quantitative PCR (qPCR). From three tested commercial kits (DNeasy PowerSoil Pro Kits, QIAamp Fast DNA Stool Mini Kit, and PureLink™ Microbiome DNA Purification Kit), the best performing one initially was DNeasy PowerSoil Pro Kits. However, after several modifications and improvements, the QIAamp Fast DNA Stool Mini Kit was the best-performing kit.

Two primer pairs were tested, one for a highly conserved *gidA* gene representing the origin of replication and one that binds to the *dcp* gene, representing the replication terminus region. A series of experiments determined that the primers are not only specific for *E. coli*, but also for *Klebsiella* and potentially other *Enterobacterales*. In conclusion, although this work did not meet its objective of developing a qPCR method specific for *E. coli*, it has identified the best-performing DNA extraction kit and established the qPCR methodology for future work, particularly an optimal primer pair that specifically targets *E. coli* and no other *Enterobacterales* species, is identified.

1. Introduction

The gut microbiome is a complex community of microorganisms found in the gastrointestinal tracts of animals. This diverse ecosystem includes bacteria, archaea, viruses, fungi, and protozoa, and is essential in maintaining health by aiding digestion, synthesising vitamins, and protecting against pathogens. Among the bacterial members, *Escherichia coli* (*E. coli*) is one of the most studied microorganisms due to its dual role as a typical gut inhabitant and opportunistic pathogen.

E. coli, a versatile bacterium with various strains, is a fascinating subject of study. Some strains of *E. coli* are harmless commensals, contributing to the microbiome's balance and aiding in nutrient absorption and the production of vitamin K. However, it's important to note that pathogenic strains can cause severe foodborne illnesses, leading to symptoms such as diarrhoea, abdominal cramps, and, in extreme cases, kidney failure. *E. coli's* potential for both good and harm is what makes it an exciting area of research among the already fascinating gut microbiome. Accordingly, this project aimed to develop a method to quantify *E. coli* in the gut microbiome.

Researchers often start by extracting DNA from faecal samples to study the gut microbiome. This process involves several steps to ensure the purity and integrity of the DNA, which is crucial for downstream applications like quantitative PCR (qPCR).

The microbial cells in the faecal sample are broken down in the first step. This can be achieved using a combination of mechanical lysis, such as bead-beating, chemical lysis buffers, and enzymes. The goal is to release and access all the DNA in the sample by breaking down the microbial cells. After the cell lysis step, the mixture contains DNA, cell debris, and potential inhibitors of downstream applications. Various purification methods, such as spin columns, magnetic beads, or alcohol precipitation, can be used to isolate and purify DNA. These methods can clean and separate the DNA from proteins, lipids, and other contaminants, ensuring the DNA is high-quality for subsequent analysis.

Quantitative PCR (qPCR) is a powerful technique for quantifying specific DNA sequences, making it ideal for measuring the abundance of individual species, such as *E. coli*, in faecal samples. The primers are designed to target regions of the *E. coli*

genome that are unique and different from other bacteria. These primers ensure that the qPCR amplifies only the *E. coli* DNA, providing specificity to the assay.

In conclusion, studying the gut microbiome and quantifying *E. coli* using DNA extraction from faecal samples and qPCR could provide insights into gut health and disease. This process involves standardised sample handling, careful DNA extraction, and accurate quantification techniques.

In this thesis, I focus on establishing a protocol for DNA extraction and attempting to quantify the amount of *E. coli* by using qPCR.

2. Background

2.1 Gut microbiome in health and disease

The human lower gastrointestinal tract hosts a diverse microbial community called the gut microbiome (**Table 1**). The gut microorganisms collectively confer several functions essential for maintaining gut and overall physiology homeostasis (Fierer et al., 2012). Some of the main functions that the gut microbiome contributes to human physiology are 1) training the immune system, 2) protecting against invading pathogenic microbes, 3) supporting epithelial barrier integrity, 4) assisting in digestion and lipid metabolism, and 5) production of bioactive compounds such as short chain fatty acids (SCFA) and vitamins (Bik et al., 2018; Klaassen & Cui, 2015; Laukens et al., 2016). The involvement of the gut microbiome in the functions essential to human physiology highlights the multiple implications for health and disease. Several research works mentioned that the adult gut is important a diverse and well-balanced microbial community, which exists in a symbiotic relationship with the host and is resistant to change (Hou et al., 2022; Zheng et al., 2020).

Table 1 *Definition: Microbiome and Microbiota (Berg et al., 2020).*

Microbiome	Characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties**.**
Microbiota	The assembly of microorganisms belonging to different kingdoms (*e.g.*, Bacteria, Archaea, Protozoa, and Fungi).

The terminology used in this thesis is based on (Berg et al., 2020), who describe the gut microbiota as all the microbes living in the gut. On the other hand, the gut microbiome means the microbiota as well as the metabolites, genetic material, macro-molecules, and environmental conditions (**Figure 1**).

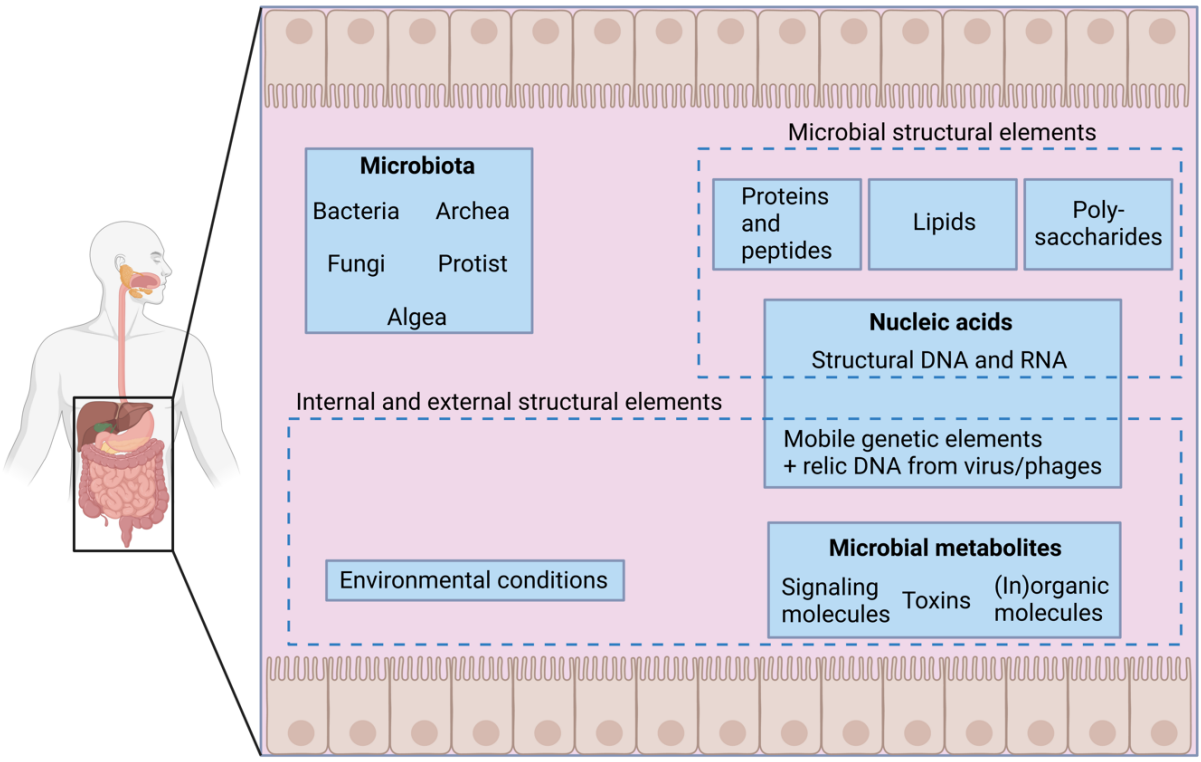


Figure 1 *Microbiome definition. Adapted from Berg Rybakova et al. 2020 (Berg et al., 2020). Created in Biorender.com*

2.1.1 Factors influencing gut microbiota composition.

The composition of the gut microbiome is highly individual and influenced by various factors, such as diet, lifestyle, medical use, age, physical activity as well as sleeping patterns. A lifestyle with ample physical activity and a healthy diet has been shown to contribute to increased microbial diversity while reducing pro-inflammatory cytokines (Rojas-Valverde et al., 2023), the following sections discuss the most prominent factors influencing the gut microbiome's composition and function.

Geography

Geography influences gut microbiota and its composition through various factors, such as the dietary habits of the populations in the area and what type of food is available. The environmental exposure to microorganisms is different in an urban setting compared to that of a rural environment and can shape the gut microbiome

already from a young age (Ahn & Hayes, 2021). Social behaviour, cultural practice, local hygiene, sanitation, and healthcare practices change exposure to microorganisms and affect people-to-people microbial exchange (Ahn & Hayes, 2021; Finlay et al., 2021). For example, genetic adaptations towards the geographical location over thousands of years can influence the gut microbiome composition directly by altering host physiology, or indirectly by affecting food choice based on intolerance (Cahana & Iraqi, 2020). One instance of such adaptation is the lactose intolerance. Where the enzyme lactase drastically decreases in levels after early childhood. Lactase is an enzyme which aids in the digestion of lactose from dairy products. Approximately 2/3 of the human population worldwide loses the ability to digest dairy products while the remaining 1/3 will have lifelong persistence (Anguita-Ruiz et al., 2020). This phenotype of lactase persistence is more common in the northern European population than other parts of the world (Gerbault et al., 2011). If the gut microbiota is efficient at hydrolysing lactose into glucose and galactose, an individual will experience less osmotic shock but at the cost of increased gas from fermenting of glucose and galactose (Lomer et al., 2008).

Diet

A nutritionally balanced diet is not just beneficial; it's crucial for maintaining a healthy gut microbiome and, consequently, the integrity of the intestinal barrier, immune tolerance, and normal gut physiology. However, an unbalanced diet, such as the typical Western diet pattern, poses a hidden risk. Pre-packaged foods, refined grains, red and processed meats, and other animal products like high-fat dairy, as well as high sugar intake from sweet snacks, sweets, or sugary drinks, can all contribute to reduced diversity, potentially leading to bacterial dysbiosis of the gut microbiome(Clemente-Suárez et al., 2023; Zhang, 2022).

One of the major types of dietary fibre, i.e., indigestible carbohydrates, plays a significant role in promoting gut health. They selectively feed fibre-degrading bacteria, which ferment them into short-chain fatty acids (SCFAs). These SCFAs are not only beneficial for gut health under normal conditions but also serve as a vital energy source for the colonocytes (Clausen & Mortensen, 1995).

Undigested proteins, however, promote the growth of proteolytic bacteria. While the proteolytic bacteria can produce SCFAs, they also produce unwanted metabolites, such as ammonia and hydrogen sulphides (Bartlett & Kleiner, 2022; Zhang, 2022).

Hydrogen sulphides are directly toxic to intestinal epithelial cells(Figliuolo, dos Santos et al. 2017), while ammonia is linked to hepatic encephalopathy(Chen et al., 2021).

The human body secretes bile acid in response to eating dietary fats. Bile acid causes selection towards bile acid-tolerant bacteria, which produce toxic compounds like H2S. As bile acids are secreted, they can conjugate into conjugated fatty acids. About 5% of the conjugated fatty acids reach the colon, where bacteria metabolise them (Singh et al., 2017; Staley et al., 2017).

A lack of fibre can have long-lasting detrimental effects on the gut microbial ecology. In mice with human microbiota, a fibre-deficient diet leads to a significant reduction in microbial diversity. Beyond supporting microbial diversity, adequate dietary fibre maintains the integrity of the mucus barrier, reducing the risk of infection from pathogens (Singh et al., 2017; Zhang, 2022).

A low-fibre diet sets the stage for the growth of colonic mucus-degrading bacteria. These bacteria, in turn, erode the intestinal mucus barrier, increasing the risk of pathogen invasion. The reduction in mucus thickness, a direct result of a fibre-free diet, brings luminal bacteria closer to the intestinal epithelium. This triggers a cascade of host responses and altered immune response pathways in the tissue (Desai et al., 2016). Enteric *E. coli* infection, combined with a fibre-deficient microbiota, allows the pathogen greater access, resulting in broader areas of inflammation in colon tissue and leading to colitis (Desai et al., 2016). Therefore, dietary fibre, obtained from varied food intake, is important in maintaining microbiota diversity and a healthy gut.

Medication use

Medications, especially antibiotics, have immense effects on microbial diversity. The use of antibiotics leads to an overall decline in bacterial diversity and causes unnecessary disruption of gut homeostasis (Patangia et al., 2022; Ramirez et al., 2020). Disruption of the gut microbial community by antibiotics leads to dysbiosis, a state of imbalance of the gut microbiota community. This imbalance involves an overgrowth of unwanted bacteria, leading to an increase in harmful bacteria and, simultaneously, a reduction of beneficial bacteria (Ramirez, Guarner et al. 2020;. However, it is not only antibiotics that can affect the gut microbiome, but also others.

For example, proton pump inhibitors that are used in the management of symptoms of several acid and reflux-related disorders. By reducing the stomach acid, it can alter the natural barrier of the gut, where long-term use can increase the risk of developing dysbiosis (Imhann et al., 2016; Jackson et al., 2016).

Disease states

Alterations in the gut microbiome, a.k.a. dysbiosis, have been associated with several diseases and conditions, including inflammatory bowel disease (IBD), obesity, type 2 diabetes mellitus, coeliac disease, colorectal cancer, as well as anxiety, depression and cognitive disorders (gut-brain–axis) (Carding et al., 2015). For example, IBD which have links to harmful Proteobacteria, which have been described to enhance the inflammatory response. Some strains of pathogenic *E. coli* strains in particular have an important role in IBD pathogenesis (Baldelli et al., 2021).

This master's thesis focuses on *E. coli*, a species from the Enterobacerales order, at the taxonomic level of species. More about *E. coli* is in **Chapter 2.3**. This bacteria order, together with *E. coli*, is one of the largest groups of bacteria responsible for severe infections with resistance to antimicrobial medications (Ren et al., 2022).

2.1.2 Stability and composition of the gut microbiota across the life span

During human life, the gut microbiota composition changes. Foetuses harbour a near-sterile gastrointestinal tract up until the point of birth. After birth, a microbial community starts to establish in the gastrointestinal tract. Since birth, the gut microbiota composition is quickly and dynamically developing, shaped by many elements in early life.

Shortly after birth, gut colonisation is dominated by bacteria from the genus *Bifidobacterium* in breastfed infants. Over time, novel food is introduced to infants besides human milk, causing increased gut microbiota diversity and a decline in *Bifidobacterium* domination. During early childhood, as the food variety increases further, microbiota diversity also increases. Shifting towards an adult-like composition happens in parallel to a diet shift resembling an adult diet. The adult-like composition allows for a broader range of dietary substances to be broken down and utilised. This contributes to metabolic health, immune regulations, and disease prevention. This, in turn, supports the host's health and resilience by aiding in maintaining gut homeostasis (Lozupone et al., 2012; Ragonnaud & Biragyn, 2021).

In adults, the microbiota composition stabilises. The two main phyla *Bacillota*, and *Bacteroidota are dominant* (formerly known as *Firmicutes* and *Bacteroidetes (Oren & Garrity, 2021))*. Phyla *Actinomyceota*, *Psaudomonadota*, and *Verrucomicrobiota* (in order: *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobi (Lozupone et al., 2012; Oren & Garrity, 2021)*) are also part of the colonisation, but to a lesser extent (Ragonnaud & Biragyn, 2021).

While diet remains a major factor in microbiota composition, the gut microbiota in elderly individuals reflects changes both from the ageing process and the impact of a long life, dietary habits, and health conditions. During the ageing process from adult to elderly, the gut microbiota composition undergoes a transition where several bacteria groups are reduced. Decreasing commensal gut microbiota allows for a relative increase in opportunistic pathogenic bacteria (Salazar et al., 2023). The gut microbiota of the elderly is characterised by a reduction of *Bacillota*, and an increase in *Psaudomonadota* (Salazar et al., 2017).

2.2 Stool as a proxy to study gut microbiota composition

The most common way for researchers to study gut microbiota is to extract DNA from faecal samples. Faecal samples can serve as a proxy for the gut microbiome in the distal colon. The popularity of faecal samples comes from the fact that they are non-invasive and easy to collect compared to alternatives such as mucosal biopsies. At the same time, they are a cost-effective method to gather samples. Faecal samples provide a broad overview by capturing a wide range of gut microbes residing in the gut. However, the main critique of this approach comes from its limit of bringing data from only the distal colon; another point of critique is the uncertainty of equal distribution of microbes throughout the faecal sample (Swidsinski et al., 2008). Donaldson et al 2016 describe the importance of mucosal biopsies, as the gut microbiome in samples at different gut locations can have significant variability (Donaldson et al., 2016).

Despite this, faecal samples remain the most popular way for researchers to study the gut microbiome. Their ease of use for volunteers, non-invasiveness, scalability, cost-effectiveness, and standardisation make them superior to other methods. Still, several considerations must be made before using stool samples for a gut microbiota analysis, which are discussed below.

Complexity of stool

Stool typically contains a high amount of water. Depending on the amount of fibre intake, the total water content for an average adult is 60 – 80% of the total faecal mass. The 40 – 20 remaining percentages of dry matter consist of undigested food, macromolecules (such as proteins, glycosaminoglycans, fibres, and DNA), and small molecules, mucus and epithelial cell shedding, as well as biomass from gut microbes, which make up 25 – 54% the dry matter (Rose et al., 2015). Studies have shown that varying water content has a correlation with the bacterial diversity of faecal samples. In 2015, Vandeputte showed that using the Bristol stool scale, the stool that was more watery stool, had lower diversity compared to that of less watery stools. Ingestion of dietary fibre (Vandeputte et al., 2016). Increasing intake of soluble but nonfermentable fibre (i.e. psyllium) may relieve symptoms of watery stool and has for a long time been used as a treatment for diarrhoea and irritable bowel syndrome (Belknap et al., 1997; Kaewdech et al., 2022; Kumar et al., 1987).

Considerations on stool collection for the gut microbiome analysis

Collecting and storing stool samples brings challenges, including the potential remoteness of donors' location, time of bowel movement, sample storage conditions, and speed of delivery to the lab. Many of these parameters affecting the sample quality can be out of control for the investigator, as well as variables associated with the stool sample itself, such as exact information on the diet, medication, and health of the donor. Another challenge is the lack of standardised collection and storage of stool samples. Method standardisation is recommended to avoid bias from contamination, changes in bacteria composition, and DNA degradation over time. The STORMS checklist aims to standardise multidisciplinary reporting and provide guidance for complete reporting of microbiome studies while still being brief and to the point (Mirzayi et al., 2021). Collected stool may be stored at different temperatures before being handed over to the downstream analyses. Moreover, the presence of metabolically active microbial cells within faeces makes their analysis prone to variations due to different collection techniques, as exposure to air and fluctuating temperatures modify the microbial composition in these samples. Employing ethanol preservation represents a viable option when immediate freezing is unattainable. Studies have shown that samples maintained in 95% ethanol for up to four days retain a metabolic profile akin to that of fresh samples (Pettersen et al., 2022).

Stool samples may also contain substances that inhibit downstream analysis, such as phenols, EDTA, or carbohydrate contamination. They must be carefully handled to avoid creating data inaccuracies. Purifying DNA and removing these inhibitors requires extra sample-handling steps.

Stool samples are often stored in a frozen state. However, if the samples are not stored correctly, it will greatly affect the sample. The bacterial composition will be altered, and the quality of genetic material will decline. Many bacteria present in the gut are sensitive to changes in temperature, moisture, and oxygen levels. Sensitive bacteria will die off, while others that are more resistant to change will still multiply, and this can cause an inaccurate representation of the gut microbiome at the time of collection. Degradation of genetic material also occurs from the time of collection. Several cycles of being frozen and repeatedly thawing are key factors in DNA

degradation. Transporting the sample at a cooled temperature may increase acceptable transportation time, but it should still be kept to a minimum.

In ideal conditions, the sample should be transported and stored as soon as possible at subzero conditions ranging from -20^{o}C to -80^{o}C. However, it's impossible to control a donor’s bowel movements, which means researchers must depend on the study participants to send the samples through the post system or personally deliver them.

DNA stabilisers can also be added to improve the preservation of DNA. This can be useful when a sample donor is unwilling to store the sample in the home refrigerator or lives in a remote location. The DNA stabilisers are buffers designed to slow down or stop DNA and RNA molecules from breaking down after sample collection. Multiple stabilisers are available on the commercial market, protecting DNA and RNA against degradation for days to weeks at room temperature. A cheap alternative to the commercial buffers is 95% ethanol. The preservation abilities of ethanol come from its strong denaturing effect on proteins, which leads to inhibition of DNAase present in the sample.

2.2.1 Relative quantification of the gut microbiota

Relative quantification is valuable for researching the gut microbiota and its composition and diversity. Relative quantification lets researchers explore the ratios of different bacterial species in a population. The technique used in exploring the relative quantity is next-generation sequencing (NGS), which is a technology that has advanced fast since the early 2000s, and it’s now possible for even small labs to sequence comprehensively and obtain high-quality data in a short time. However, only relative quantitative data can be derived from NGS data because of the instrument's limits. This limits the NGS acquired data to be compositional. Therefore, ecologically, only the relative abundances of bacteria in a sample are possible to investigate. The problem arises when the absolute quantity of one bacteria species is not influenced by the other bacteria. Therefore, only knowing the compositional data from the gut microbiome can mask the total microbial load and density(Gloor et al., 2017).

2.2.2 Absolute quantification of the gut microbiota

Cell-based and molecular-based methods both make it possible to analyse the same datasets for absolute quantities of bacteria. Flow cytometry (FCM) has previously been used by Vandeputte et al. to determine microbiome cell counts in faecal samples (Vandeputte et al., 2017). DNA-or cell spike-in has been used to estimate the absolute abundance in microbiome NGS data sets (Stämmler et al., 2016). As well as, total DNA extraction yield has been used for estimating gut microbiota density and load (Contijoch et al., 2019) (Korpela et al., 2018). Quantitative Polymerase chain reaction (qPCR) is another molecular-based method that can be used for quantifying gut microbiota (Haugan, Charbon et al. 2018) and may have some advantages over FCM and a spike in methods (Jian et al., 2020).

Flow cytometry

Flow cytometry (FCM) is a laboratory technique routinely used in research to analyse cells, including bacteria, using a cytometer instrument. The instrument can include a cell counter for bacteria enumeration, assessing cell viability, sorting, and isolating cells based on their different cell characteristics. Because of this, it can potentially be used for studies about microbial ecology, measuring diversity in a sample (Chen & Cherian, 2017; Midani & David, 2023).

FCM works on three systems: a fluidic system, an optical detection system and an electronic system (**Figure 2**). The fluidic system directs the bacterial cells into the focused light. The optical system then focuses the light onto the bacterial cells and collects information about the light scatter and fluoresces. The electronic system then converts this information into useful data. If the FCM machine is equipped with a cell sorter, the data allows the FCM to separate the stained and unstained bacteria from one another in a sample with a mixed population (Adan et al., 2017).

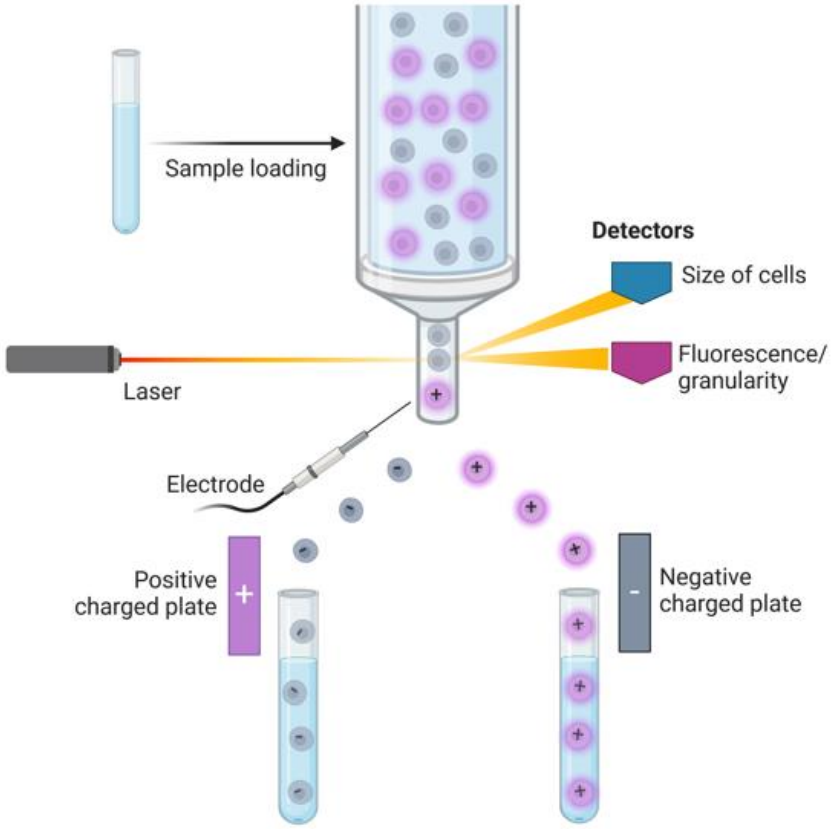


Figure 2 *Flow cytometry instrument. Cretated in Biorender.com*

qPCR

PCR is a common and powerful laboratory technique for assessing the presence of genes. Short synthetic DNA fragments, called primers, target the gene of interest, creating an amplified DNA product in a three-step process: Denaturation, annealing, and extension (**Figure 3**). Repeated cycles of the three-step process led to a massive increase of copies (amplification) of the targeted gene. And after 30-40 cycles, creating millions to billions of copies of the amplified gene product(Sigma Aldrich, 2024a).

Quantitative PCR (qPCR) adds another layer by introducing fluorescent dye, making it able to quantify the number of bacteria in a sample. qPCR determines the number of products present in each PCR cycle by using a fluorescent dye that binds to the double-stranded DNA after each cycle. When the dye binds to any of the double-stranded DNA in the reaction, the dye will start to fluorescent. As the target gene is amplified $x*2^{40}$ time, the fluorescent dye allows the detection of new DNA in each generation with cumulatively increased fluorescence after each sequent qPCR cycle. Based on cycle number and fluorescence, the qPCR can track the target bacteria

DNA concentration as it increases and, with this information, calculate the original concentration of bacteria DNA that was in our sample at the start (Sigma Aldrich, 2024b). For an overview of the qPCR and its steps, see **Figure 3** below.

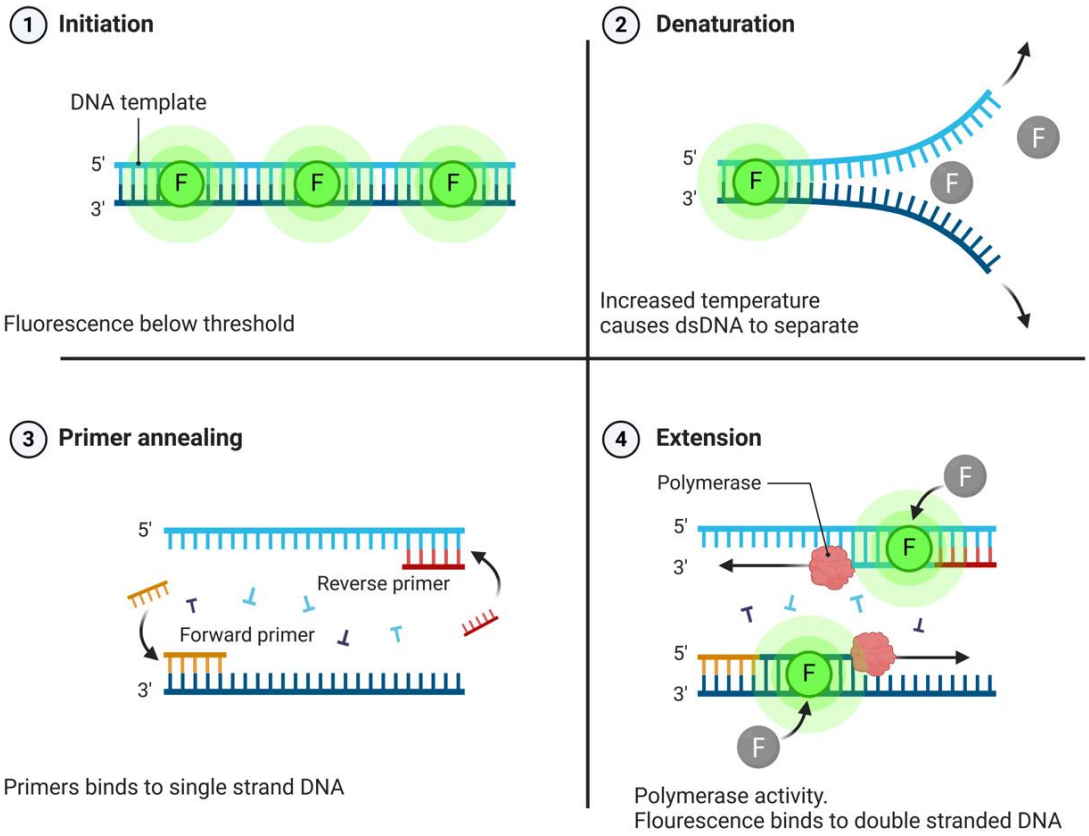


Figure 3 qPCR overview Created in Biorender.com

DNA/Cell Spike-in

The third method, DNA Spike-in or Cell Spike-in (Spike-in), is used for faecal microbiota quantification. By adding a known amount of DNA to samples to be used as a reference value.

To evaluate the performance of analytical methods used when researching gut microbiota, a known substance in known quantities can be added at various stages of the sample-handling process. This aims to bring normalisation, quality control and validation. By adding synthetic DNA or mock bacteria communities to the sample, one can validate the method's accuracy and consistency and offer a quantitative reference for comparison in a quantitative method. For example, by spiking in the sample before DNA quantification, one can ensure the efficacy of the method used and compare/benchmark it against other methods.

Comparison of FCM and qPCR methods for faecal microbiota quantification

While FCM lets one to separate and enumerate live bacterial cells, it also has drawbacks that make other methods more suitable. Running a project based on FCM is expensive. FCM requires trained personnel to use sophisticated equipment. qPCR, on the other hand, is a simple and cost-effective method that can be done in most labs. Tools and reagents used for running a qPCR project are similar to those of NGS and can used in both processes (Jian et al., 2020). It is also easier to scale qPCR up to high throughput because of the 96 and 384 well formats.

While both FCM and qPCR can produce precise results with high correlation when used to quantify microbiota abundance, the two methods produce highly diverse microbiota profiles when quantifying the composition in stool samples (Galazzo et al., 2020).

Both FCM and qPCR provide precise results and can both be used to quantify bacteria. FCM offers detailed analysis capabilities, such as differentiating between live and dead cells, but it requires significant expertise. The equipment and reagents needed are often more expensive, and sophisticated instruments are necessary for high-throughput setups. While flow cytometry can provide detailed insights, it requires careful use of controls and a substantial amount of data transformation.

On the other hand, qPCR is relatively simple, requiring no complex controls or data transformations. While FCM can differentiate between bacteria cells, they must be intact. However, qPCR is a molecular method targeting bacterial DNA. Additionally, qPCR is still effective in samples with high host or non-bacterial DNA content with its selectively binding primers .

The advantages of qPCR's accessibility and ease of use make it a perfect option for a master's project like this. For an overview of the Advantages and drawbacks of different methods of quantifying gut microbiota, see **Table 2** below.

Table 2 *Comparison of methods for microbiota quantification*

	Advantage	Drawbacks
Flow Cytometry	Single-cell resolution. Can differentiate between live and dead bacteria.	No high-throughput method. Formation of aggregates. Fresh sample needed. Technically demanding, less specific. Extra lab process.
qPCR	Simple and cost-effective. Similar lab setups to NGS. It can be used to quantify other microbes as well.	Requires DNA extraction and can be influenced by PCR inhibitors. Copy number variation. Lower sensitivity. Extra lab process.
DNA or cell spike in methods	Comparability to NGS. Applicability. Specific Part of the sequencing process Provides internal control for normalisation, calibration, and validation.	Bias in sample preparations. Copy number variations.

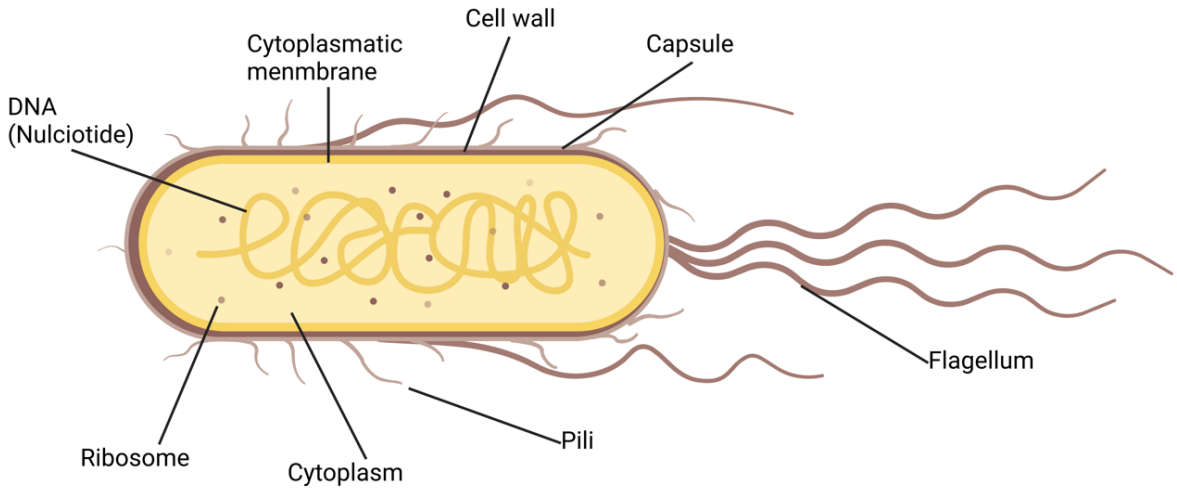
2.3 *Escherichia coli*, a versatile bacterial species

Escherichia coli is a rod-shaped, gram-negative facultative anaerobic bacterial species that is commonly found in the gastrointestinal tract of humans and other warm-blooded animals. *E. coli* belongs to the bacterial order *Enterobacterales*, which lies under the phylum *Pseudomonadota*. This bacterial order is closely linked to disease due to the presence of many pathogens or opportunistic pathogens attributable to gastrointestinal infections (Raffelsberger et al., 2021). The infection-transmission route is often the fecal-oral route by ingesting contaminated food or water (Veterinærinstituttet, 2023). Their pathogenicity is frequently exacerbated by their ability to acquire resistance to multiple antibiotics, which makes it difficult to treat their infections (Ordonez et al., 2021). Antibiotic resistance and a wide range of diseases, cause pathogenic bacteria from the *Enterobacterales* order to pose a significant challenge in healthcare settings (Ordonez et al., 2021).

E. coli display large genomic diversity, with different nutrient preferences between commensal and pathogenic strains (sub-species level resolution) (Conway & Cohen, 2015). Various *E. coli* strains can be beneficial and coexist within the host digestive system (Nakkarach et al., 2020; Siniagina et al., 2021). Commensal strains of *E. coli* aid digestion and nutrient absorption by breaking down and fermenting complex carbohydrates that are otherwise indigestible for the human host. These biosynthesis products and fermentation products are important bioactive compounds that are beneficial to the host and vital to its physiology. For example, the two vitamins, vitamin K and B12, are products of *E. coli* biosynthesis (Blount, 2015). Some strains

have also been found to produce short-chain fatty acids (SCFA), like strain *E. coli* Nissle 1917, which is used in probiotic supplements (Nakkarach et al., 2020; Wassenaar, 2016). These SCFAs act as an important energy source for the colonocytes. Studies have shown that as much as 60-70% of the colon's energy needs are covered by oxidation of SCFA produced in the digestive tract by gut microbes (Den Besten et al., 2013).

Figure 3 *Illustration of E.coli cell. Cretated in Biorender.com*



E. coli also serves an important function in preserving a healthy gut environment by preventing pathogenic microbes from colonising. By exclusion through competition of available nutrients and ecological niches, *E. coli* limits harmful bacteria from getting a foothold and establishing colonies inside the intestinal tract.

It is important to note that while most *E. coli* strains that inhabit the gastrointestinal tract are commensal and seldom cause disease, *E. coli* also happens to be one of the most frequent causes of common bacterial infection. This indicates that there is a thin balance between *E. coli* and its human host to maintain a healthy gut. The distinction between commensal and pathogenic *E. coli* lies in their strain-specific characteristics. For example, pathogenic strains possess specific virulence factors, such as toxins and adhesins, which aid the pathogenesis process.

Transmission and infection of *E. coli* typically occur when consuming contaminated food or water. Inadequate hygiene and handling during food processing and cooking are usually the source of *E. coli* transmission. Examples are contaminated meat, raw vegetables, and unpasteurised dairy products.

Antibiotic resistance is also a growing concern with *E. coli*. *E. coli* is known to be able to acquire antimicrobial resistance genes (ARGs). Due to mobile genetic elements (MGEs), horizontal gene transfer has caused the diffusion of ARGs among *E. coli* and other bacteria species. These MGEs consist of plasmids, transposons, and insertion sequences and are part of the adaptability of the *E. coli* genome. In a high population, high-density areas like the intestinal tract, where the bacterial diversity is also high, sharing of ARGs between the commensal species might be common, although not yet proven. Because of this, *E. coli* has been proposed as a sentinel microorganism for overall antimicrobial resistance {Washington, 2021 #300}. Particularly in regards to beta-lactams, but also for antimicrobial resistance in general (Ramos et al., 2020){Nyirabahizi, 2020 #278}.

2.3.1 *E. coli* levels in infancy and adulthood

In the early life of neonates, rapid population and colonisation of gut microbiota occur, among them *E. coli*. During infancy, *E. coli* emerges as one of the first colonisers in the infant's gut and has an important role during the initial maturation of the immune system, as well as protecting against colonisation by opportunistic pathogens (Han et al., 2024; Jian et al., 2021). Early colonising *E. coli* strains are thought to have been acquired both from the mother and the environment. In infancy and throughout childhood, diet rapidly changes with age until adulthood, where diet, lifestyle factors, and microbiota composition stabilise {Rinninella, 2019 #299}.

2.3.2 *E. coli* as a carrier of AMR genes

In normal conditions, *E. coli* is a commensal bacterium in our gut. *E. coli* aids with digestion and protects against the colonisation of opportunistic pathogens. However, *E. coli* is also one of the top causes of bacterial infections and has been labelled as one of the six main pathogens associated with death related to antimicrobial resistance. Resistance to antimicrobial agents, for example, antibiotics, is a major global burden(Murray et al., 2022). Infections from bacteria with antimicrobial resistance are becoming increasingly prevalent in the newborn intensive care unit and are difficult to treat with the two main pathogenic species in infants being *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*). ¨

Within the group of bacteria that carries AMR genes, *E. coli* serves indicator of AMR within the hosts. *E. coli* is found in all warm-blooded animals, it is therefore found in

livestock used in food production. *E. coli* has flexible and adaptable genetics that allow it to acquire a great number of mechanisms against antimicrobial techniques by harbouring many AMR genes.

Mobile genetic elements contribute to *E. coli*'s strong adaptability. *E. coli*'s ability to potentially transfer resistance genes both to other *E. coli* bacteria and to other commensal bacteria species has led to it being considered an indicator microorganism for AMR surveillance, for example, for beta-lactams (Nyirabahizi et al., 2020). *E. coli* contamination in food, faecal-oral route, and link to increased AMR carriage in the gut

3. Objectives

Description of the gut microbiome by relative abundances has been shown to be sometimes inaccurate and potentially lead to false findings. Methods for absolute quantification of microbial DNA can remedy these limitations by determining the actual number of microbial cells. The aim of this thesis work was to develop and optimise a protocol for absolute quantification *of E. coli* from stool samples. *E. coli* is an important human gut commensal as well as an opportunistic pathogen causing severe illnesses. Because of the clinical relevance, it was chosen as the model microorganism for this project.

This project first established an optimised method for extracting bacterial DNA from stool samples and secondly, attemted to quantify the amount of *E. coli* in extracted DNA from human stoool.

4. Material and Methods

4.1 Materials

Table 3 *List of materials*

Product	Catalog number	Manufacturer
Lying matrix E 2mL	116914050-CF (MP Biomedicals, 2024)	MP Biomedicals, Santa Ana, CA
Ethanol absolute		
Duran flask		DURAN Group GmbH, Wertheim, Germany
Mili-q water		
HyClone HyPure Water, Molecular Biology Grade	SH30538.LS (Cytiva, 2024)	Cytiva life sciences, Marlborough, USA
DNA LoBind® Tubes 1,5mL	0030108051	Eppendorf, Hamburg, Germany
SafeSeal reaction tube, 1.5 ml, PP, PCR Performance Tested, Low DNA-binding	72.706.700	SARSTEDT AG & Co. KG, Nümbrecht, Germany
Falcon tube 15mL conical tube		
Ambion™ RNase-Free Conical Tubes 15 mL	AM12500	ThermoFisher Scientific, Waltham USA
ART™ Barrier Pipette Tips in Lift-off Lid Rack 0,5-10µL	2149	ThermoFisher Scientific, Waltham USA
ART™ Barrier Pipette Tips in Lift-off Lid Rack 100µL	2065E	ThermoFisher Scientific, Waltham USA
ZAP Premier stenle aersosol pipet tips 1-100µL	732-1102	Labcon, California, USA
ART™ Barrier Pipette Tips in Lift-off Lid Rack 200µL	2069	ThermoFisher Scientific, Waltham USA
ART™ Barrier Pipette Tips in Hinged Racks 1000µL	2179-HR	ThermoFisher Scientific, Waltham USA
Takyon Low ROX SYBR MasterMix dTTP Blue	UF-LSMT-B0701	Eurogentec, Belgium
T4 Gene 32 Protein	M0300S	new england biolabs inc. Massachusetts, United States
MicroAmp Fast Optical 96-well reaction plate	4346906 (Thermo Fisher Scientific, 2024b)	ThermoFisher Scientific, Waltham USA
LightCycler 480 Multiwell Plate 96	04729692001 (Roche Life Science, 2024)	Roche Applied Science Penzberg, Germany
Qubit™ 1X double-stranded DNA high-sensitivity kit	Q33231 (Thermo Fisher Scientific, 2024d)	ThermoFisher Scientific, Waltham USA
Primer FWD gidA	n/a	Sigma-Aldrich, St. Louis, Missouri, United States
Primer REV gidA	n/a	Sigma-Aldrich, St. Louis, Missouri, United States
Primer FWD dcp	n/a	Sigma-Aldrich, St. Louis, Missouri, United States

Primer REV dcp	n/a	Sigma-Aldrich, St. Louis, Missouri, United States
Primer FWD U16S	n/a	Sigma-Aldrich, St. Louis, Missouri, United States
Primer REV U16S	n/a	Sigma-Aldrich, St. Louis, Missouri, United States

Table 4 *List of equipment*

Product	Catalog number	Manufacturer
DNeasy PowerSoil Pro Kit	47014 (QIAGEN, 2024a)	QIAGEN N.V., Hilden, Germany
QIAamp Fast DNA Stool Mini Kit	51604 (QIAGEN, 2024b)	QIAGEN N.V., Hilden, Germany
PureLink™ Microbiome DNA Purification Kit	A29790(Thermo Fisher Scientific, 2024c)	ThermoFisher Scientific, Waltham USA
Wizard® Genomic DNA Purification Kit	A112 (Promega, 2024)	Promega Corporation, Madison, Wisconsin, USA
Precellys® Evolution Touch Homogenizer	P002511-PEVT0-A.0 (BERTIN-TECHNOLOGIES, 2024)	BERTIN TECHNOLOGIES, Montigny-le-bretonneux, France
7500 real-time thermocycler	4351104 (Applied Biosystems®, 2024)	Applied Biosystems, Life Technologies, Waltham, USA
Applied Biosystems 7500 Real-Time PCR System v2.3 software	n/a	Applied Biosystems, Life Technologies, Waltham, USA
LightCycler® 96 Instrument	05815916001 (Roche Life Science, 2020)	Roche Applied Science Penzberg, Germany
LightCycler® 96 Instrument with LightCycler® 96 SW 1.1 software	n/a	Roche Applied Science Penzberg, Germany
Laminar flow hood with UV-light	n/a	
VWR Analog Vortex Mixer	10153-840	VWR, Pennsylvania, USA
VWR MiniStar White (Centrifuge)	521-2161	VWR, Pennsylvania, USA
Centrifuge 5430 R	5428000205	Eppendorf, Hamburg, Germany
NanoDrop	ND-ONE-W (Thermo Fisher Scientific, 2015a)	ThermoFisher Scientific, Waltham USA
Qubit	Q33231 (Thermo Fisher Scientific, 2017)	ThermoFisher Scientific, Waltham USA
Thermal shake *lite*	460-0249P	VWR, Pennsylvania, USA
Incu-Shaker™ 10LR	H2012	Benchmark Scientific, Inc. Sayreville, USA
Sartorius balance		Sartorius, Göttingen, Germany
Finnpipette™ F2 Variable Volume Pipette 0,2-2µL	4642010	ThermoFisher Scientific, Waltham USA
Finnpipette™ F2 Variable Volume Pipette 2-20µL	4642060	ThermoFisher Scientific, Waltham USA
Finnpipette™ F2 Variable Volume Pipette 20-200µL	4642080	ThermoFisher Scientific, Waltham USA
Finnpipette™ F2 Variable Volume Pipette 100-1000µL	4642090	ThermoFisher Scientific, Waltham USA

Mline® Mechanical Pipettes 0,5-10µL	725020	Sartorius, Göttingen, Germany
Mline® Mechanical Pipettes 10-100µL	725050	Sartorius, Göttingen, Germany
Mline® Mechanical Pipettes 20-200µL	725060	Sartorius, Göttingen, Germany
Mline® Mechanical Pipettes 100-1000µL	725070	Sartorius, Göttingen, Germany
Thermo Scientific™ Forma™ FDE Series Ultra-Low Temperature Freezer	FDE60086LDRCO	ThermoFisher Scientific, Waltham USA
Evosafe-SERIES™ Ultra-Low Freezer -86ºC HF570-86	HF570-86G	Snijders Labs, Tilburg, Netherlands
-20 Freezer Miele		Miele, Gütersloh, Germany
-20 Freezer xx		
TVarmeskap		Termaks AS, *Kungsbacka*, Sweden
Stool Nucleic Acid Collection and Preservation System	53700	Norgen Biotek, Ontario, Canada
SevenExcellence pH meter S400	30046240	Mettler Toledo, Ohio, USA

4.1.1 Human samples

The sample used for testing efficiency of DNA extraction came from a healthy donor. It was stored in the -80ºC freezer without preservatives, such as RNAlater or ethanol. Before processing, the sample was thawed on ice and aliquoted into 37 (n=37) 1,5 mL microcentrifuge tubes. The weight of each aliquot was recorded. These aliquots were used to establish and optimise the DNA extraction and isolation protocol.

The samples used for qPCR came from healthy volunteers who participated in the Tromsø 7 study. These volunteers were re-contacted and requested to donate faecal samples as part of the longitudinal Klebsiella project (Lindstedt et al., 2022). The samples were collected by using Norgen Stool Nucleic Acid Collection and Preservation System. DNA was isolated and purified using PureLink™ Microbiome DNA Purification Kit. Samples from eight individuals (n=7) were chosen for this project to determine the limit of detection (LOD) and quantification (LOQ). These eight samples were chosen because of their low abundance of *E. coli,* which was determined by metagenomic sequencing (unpublished work Kenneth Lindstedt et al). The Regional Committee for Medical and Health Research Ethics in North Norway granted approval for the project involving qPCR analysis of faecal samples from healthy adult participants (REK North reference 137064/2020).

4.2 Methods

In this thesis, the methods are divided into two topics. The first topic involves bacterial DNA extraction with and without protocol modifications, while the second topic provides details about absolute quantification of *E. coli* in human stool samples.

4.2.1 DNA Extraction and Isolation

Extracting total DNA from a faecal sample is a common way to study the gut microbiota and a prerequisite for quantifying bacterial DNA. Choosing an appropriate DNA extraction kit to work with is important to get a high yield of high-quality DNA. In this thesis, I compared and optimised three different column-based kits for DNA extraction from human stool samples: namely DNeasy PowerSoil Pro Kit, May 2019. (PowerSoil), QIAamp Fast DNA Stool Mini Kit, December 2017 (QIAamp), and PureLink™ Microbiome DNA Purification Kit, September 2015 (PureLink). To make the standard curve for qPCR, a fourth DNA extraction kit, Wizard® Genomic DNA Purification Kit, was used to isolate genomic DNA from *E. coli*.

The spin column-based DNA extraction protocols referred to in this thesis all use repeated centrifuging of sample material. The gravitational force of the centrifuge is performed in several steps to separate the lysate and supernatant from the cell pellet, and the process passes the sample and reagents through the silica matrix filter. Most commercial kits for DNA extraction follow six main steps.

1. Bacterial cell lysis.
2. Removal of contaminants that can affect downstream applications. These include PCR inhibitors such as proteins, polysaccharides, and bile salts.
3. Binding of nucleic acid to a silica matrix by adjusting pH and osmolality.
4. Washing of the bound DNA.
5. Removal of the washing solvent.
6. Elution and recovery of DNA from the silica matrix with either a low ionic strength buffer or water.

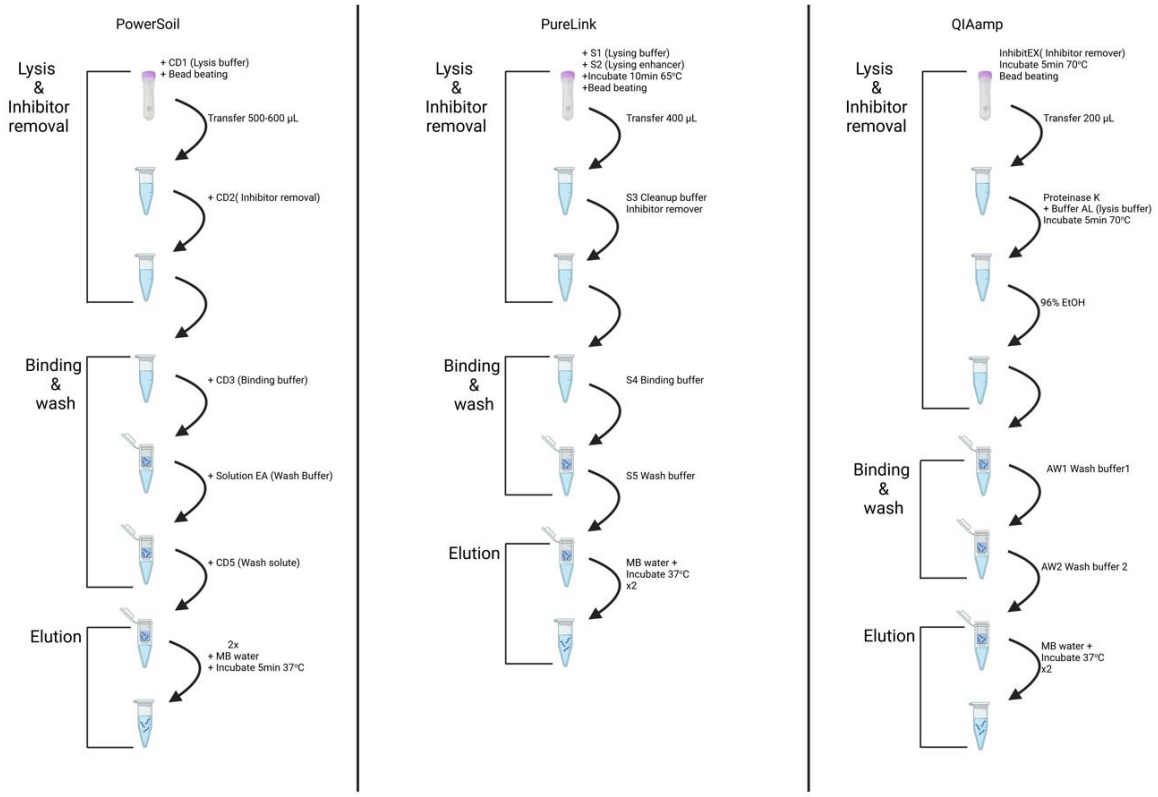


Figure 4 *Overview of the three DNA extraction kits, PowerSoil, PureLink and QIAamp Cretated in Biorender.com.*

4.2.2 Bacterial cell lysis

Common methods for bacterial cell lysis include mechanical, chemical, and thermal lysis as an initial step to release the bacterial DNA. The three selected commercial protocols apply varied combinations of methods for bacterial lysis. See **Table 5** for a detail overview and comparison of the three protocols that were optimised in this thesis.

Mechanical lysing of bacterial cells using bead-beating technology is a commonly used laboratory technique when studying the gut microbiome. Bead beating in this thesis involves the use of small beads in combination with a high-powered homogeniser to disrupt the bacterial cell wall and cell membrane with mechanical force. And will be in any referred text below be described as bead beating. This is different from the original commercial protocols using bead-beating tubes in combination with a vortex instead of a homogeniser.

Bead size, bead material, and bead geometry may have different effects on the stool sample, so it is important to choose optimal settings.

In this work, several modifications to all three protocols were adapted. Faecal sample was first mixed with ethanol (1:3 ratio in mg:µL) and placed in a screwcap 2mL tube with beads (Lysing Matrix E) along with the kit lysis buffer. The Precellys® Evolution homogeniser was then used with the following bead beating settings: 6500rpm for 2 × 23 seconds cycles, with a 30-second break between the two cycles. During the bead beating treatment, the beads collide with the cells, the cells break apart, and intracellular material, including the bacterial DNA, gets released into the lysate.

In the original manufacturer protocol, all three DNA Extraction kits a chemical lysis was introduced as the first step. Additionally, PowerSoil and PureLink included a mechanical lysis step using a bead tube with a vortex. While, PureLink and QIAamp combined to perform thermal cell lysis by incubation at high temperatures together with buffer for chemical lysis simultaneously (**Table 5**)

For thermal cell lysis PureLink heat treated the sample for 65^{o}C for ten minutes, while QIAamp heated treated the sample at 70^{o}C, first for 5 minutes, and at a later step for 10 minutes more.

To remove contaminants, after cell lysis, all DNA extraction kits utilised a clean-up buffer that binds to the contaminants, creating precipitates that can be removed by centrifugation.

In all three DNA extraction kits, the supernatant was transferred to a silica filter spin column for DNA binding. QIAamp added high-concentrate ethanol to the supernatant to bind the DNA to the filter, making the DNA aggregate. In the PowerSoil and PureLink protocols, a patented chemical complex was used as a binding buffer. After DNA binding, specific washing agents were introduced, which let the DNA stay bound to the silica filter. The centrifuge was then used to remove the remaining solvents in all three kits.

For the final step, the kits all utilised an elution buffer to aid in eluting DNA from the sample. The elution buffer increases the pH, releasing the DNA from the silica filter. For a detailed comparison, see **Table 5**. For an overview of the DNA extraction kits. The original protocols are to be found in the appendix.

Table 5 *Overview of DNA extraction protocols*

	Powersoil	PureLink	QIAamp	Modifications
Dilute	-	-	-	1:3 EtOH/MQ
Lysis	Mechanical (bead tube vortex) Chemical (CD1)	Mechanical (bead tube vortex) Chemical (S1+S2) Thermal (incubate)	Chemical (proteinase K + buffer AL) Thermal (Incubate)	Mechanical (bead beating) Chemical Thermal
Contaminants	CD2	S3	InhibitEX	
DNA binding	CD3 Binding buffer	S4 Binding buffer	96% EtOH	
DNA Washing	Solution EA	S5 Wash buffer	AW1 Wash buffer 1	
Solvent removal	CD5 Wash solute	S5 Wash buffer	AW2 Wash buffer 2	
Elution	50-100µL C6 Elution buffer	100µL S6 Elution buffer	200µL Buffer ATE	5 min incubation + 2 x 30µL H_2O

4.2.3 Optimisation of the DNA extraction protocols

An initial dilution step was added before cell lysing. The samples were diluted with eighter 95% Ethanol or Milli-Q water in a 1:3 dilution. The diluted sample was then mixed with kit-specific cell lysis buffers and underwent bead-beating as described in **Chapter 4.2.2** before incubating the sample according to the original manufacturer's kit guidelines. Note: at this point, the lysate in QIAamp, could be split into 1-3 separate tubes.

For QIAamp, the sample was transferred to a new tube with Proteinase K and buffer AL to incubate again, combining the kit's steps with chemical, mechanical, and thermal lysing of bacterial cells. PowerSoil and PureLink do not incubate after bead beating. The original manufacturer's kit guidelines for all three kits were then followed for DNA binding, DNA washing, and solvent removal.

Extracted DNA was recovered by two-step elution, including carefully pipetting 30µL of molecular biology-grade (MB) water to the centre of the silica filter. The sample was incubated for 5 minutes at 37°C, letting the silica filter and the nucleic acids bound to it get fully hydrated before centrifugation. The spin filter was centrifuged following manufacturer guidelines. The elution step was repeated to obtain a total of 60µL elute. The final protocol is presented in the results section.

4.2.4 Determination of DNA purity and quantity

Assessment of DNA purity was done by using NanoDrop One according to the in-house laboratory guidelines. Briefly, cleaning the pedestal with lint-free lens paper was followed by soaking the lens with 2µL MB water for 3 minutes and wiping the lens again with lint-free lens paper before adding the sample. The pedestal was also cleaned with MB water and lint-free lens paper between each sample that was assessed. Nanodrop detects double-stranded DNA in the range of 2 ng/µL to 15,000 ng/µL and assesses the quality in the form of the absorbance ratio in different wavelengths. The quality is then reported as the ratio between absorbance at 260 nm and at both 230 and 280 nm. Absorbance at 260 nm detects nucleic acid. Absorbance at 230 nm detects the presence of contaminants such as phenols, guanidine, chaotropic salts and carbohydrates. At absorbance 280 nm, protein is detected. Therefore, the ratio of A_{260}/A_{280} at ~1.8 is accepted as sufficiently pure DNA, and the ratio of A_{260}/A_{230} at ~2.0 is interpreted as free from contaminants (Thermo Fisher Scientific, 2015b).

In addition to NanoDrop, the Qubit® 3.0 fluorometer was used to assess DNA quantity. The fluorometer was used together with the Qubit reagent: Qubit™ 1X double-stranded DNA high-sensitivity kit. The kit includes a dye that binds specifically to double-stranded DNA (dsDNA) and emits fluorescence when binding. The fluorometer can quantify dsDNA at low concentrations (down to 10 pg/mL) and differentiate it from both single-stranded DNA and RNA in samples. The high specificity and sensitivity towards dsDNA allow the use of as small volumes as low as 1µL of the measured sample when performing a concentration analysis with Qubit.

4.2.5 Absolute quantification of *Escherichia coli*

The aim of this part of the thesis was to perform an absolute quantification of E. coli in human stool samples. For this, a standard curve was produced based on the E. coli ATCC 25922 strain. This *E. coli* strain is well-characterised and often used as a reference strain (ATCC, 2024; Minogue et al., 2014). The stain provides good comparability to other publications, and because it is a non-pathogenic strain, working with it in the laboratory is safe. The production of a standard curve is essential when compared with qPCR performed on human stool samples, as will be described later in this section.

4.2.6 Bacterial cultivation and isolation of DNA from *E. coli*

In order to isolate and test bacterial DNA from the selected *E. coli* strain, the following procedures were performed.

E. coli ATCC 25922 commercially available frozen stock was partly thawed from storage at -80ºC. The tube containing the E. coli strain was then placed on ice to avoid thawing more than necessary. A Bunsen burner was used to sterilise the metal inoculating loop; the inoculator was then used to dip into the ATCC 25922 stock sample and streaked over sterile Lysogeny broth (LB) agar plates. The LB plates were then incubated at 37ºC overnight.
From the incubated bacterial plates a single bacterial colony was then inoculated in falcon tubes containing filtrated brain-heart infusion (BHI) liquid media. One tube was left without introducing bacteria to serve as a negative control. The falcon tubes were then incubated at 37ºC overnight. All work was conducted near the burning Bunsen burner to minimise chances of contamination by airborne microorganisms.

To perform the *E. coli* DNA isolation, the Wizard® Genomic DNA Purification Kit was used. For the starting material for DNA isolation, 1mL syringe filtered inoculum from the overnight bacterial BHI culture was used. The procedure was carried out according to manufacturer guidelines for Gram-negative bacteria (ref, date Feb. 2022). The following modifications were made: All steps, including centrifugation, were done at 16.000 x g. After the heated incubation step, the protocol asks acclimatisation of samples back to room temperature. This was done using an incubator, changing temperature from 80ºC to 23ºC, and 37ºC to 23ºC. After adding isopropanol, the centrifugation time was increased to 15 minutes. The DNA pellet was rehydrated with 60µL MB water instead of 100µL of the rehydration solution included in the kit. The isolated gDNA was then used to create a qPCR stock by diluting an aliquot from 998,9 ng/µL to 49,5 ng/µL.

4.2.7 Protocol for quantitative PCR

To perform qPCR, three primer pairs were tested (Sigma-Aldrich). Two pairs were selected based on publication from Haugan et al.(Haugan, Charbon et al. 2018), and an additional universal pair targeted 16S rRNA was used as a control. See **Table 6** for details about primer names and sequence. These primers were selected based on their use by Haungan et al. and the *E. coli* ATCC 25922 reference strain.

Upon arrival, all primers were diluted with MB water before being aliquoted for a final concentration of 100µM. The primers were then stored at -20ºC until use.

Table 6 *Primer and their sequence*

Primer direction	Sequence
gidA (representing the oriC region)	
Forward primer	5´-CGCAACAGCATGGCGATAAC-3´
Reverse primer	5´-TTCGATCACCCCTGCGTACA–3´
dcp (representing the terC region)	
Forward	*5′-TCAACGTGCGAGCGATGAAT-3′*
Reverse	*5′-TTGAGCTGCGCTTCATCGAG-3′*
U16S	
Forward	*5′*-TCCTACGGGAGGCAGCAGT-*3′*
Reverse	*5′*-GGACTACCAGGGTATCTAATCCTGTT-*3′*

Each reaction mixture had a final volume of 20 µL, of which 17.5 µL of MasterMix and 2.5 µL of sample DNA/prepared standard/blanking water. In **Table 7** the mastermix reagents are listed. All DNA isolated from stool samples was diluted to 10ng/µL and run in technical quintuplets, while standard and blank were run as technical triplicates.

Table 7 *MasterMix components*

MasterMix	×1	×80
Takyon Low ROX SYBR MasterMix dTTP Blue	10 µL	800 µL
Forward primer	0,06 µL	4,8 µL
Reverse primer	0,06 µL	4,8 µL
T4	0,025 µL	2 µL
MB-Water	7,335 µL	588,4µL
Total:	**17,5µL**	**1400 µL**

The qPCR assays were performed on two different qPCR machines: a 7500 real-time thermocycler, and a LightCycler® 96 Instrument. The reason for using two different qPCR machines was a warranty expiration and loss of access mid-project.

To reduce false positive detections, acceptance criteria were as follows: Samples were required to be tested in technical triplicate (or more), have a minimum of 2/3 positive replicates and have a cycle threshold (Cq) value of fewer than 40 cycles. Primer melting temperature (Tm) was estimated in silico by using an online oligo analysis tool (Genomics, 2024) Eurofins Genomics, Ebersberg, Germany): An

acceptable temperature range before suspecting primer mishybridisation was decided to be Tm +- 1°C. See **Table 8** below for an overview of the primers melting temperatures. Cycling conditions for qPCR are described below in **Table 9**.

Table 8 *Primer melting temperature.*

Primer	Tm	Lower-end Tm	Higher-end Tm
gidA	87°C	86°C	88°C
dcp	88°C	87°C	89°C
U16S	90°C.	89°C	91°C

Faecal background interference can affect the melting temperature of primers, therefore, a range for acceptance is permitted.

Table 9 *qPCR cycling conditions*

	Temperature	Time	
Holding	95ºC	00:30	Enzyme activation
Cycling x40	95ºC 58ºC	00:05 00:30	Denaturation Annealing (data collection)
Melt curve	95ºC 60ºC 95ºC	00:15 01:00 00:15	Dissociation stage Program step and hold T increment +0.3C

Special care was taken during preparing the reaction mixes. All pipetting was done with filtered tips, and the microcentrifuge tubes used were DNA-loBind. MB-water was aliquoted to RNAse-free Falcone tubes prior to the experiment to avoid potential contamination in future experiments. 96-well reaction plates (0.1 mL) were used for qPCR. For Applied Biosystems 7500 Real-Time PCR, a clear polypropylene MicroAmp Fast Optical 96-well reaction plate was used. For the LightCycler® 96 Instrument, a white polypropylene LightCycler 480 Multiwell Plate 96 was used.

4.2.8 Calibration of the qPCR standard curve

The isolated genomic DNA (gDNA) from *E. coli* strain ATCC25922 was used to construct the standard curve that could allow for DNA quantification in stool samples. This was done by creating a series of 1:5 dilutions from 250,000 genomes/reaction down to 3,2 genomes/reaction. Specifically, seven sets of five-fold dilutions of *E. coli* gDNA with final genome copies per reaction of 250 000, 50 000, 10 000, 2000, 400,

80, 16 and 3,2 were used for the standard curve **Table 10**. Genome copies per reaction were calculated using the equation below (Clifford et al., 2012).

$$Genome\ copy\ number = \frac{(Mass\ of\ input\ DNA\ in\ ng) \times (6.022 \times 10^{23}\ molecules/mole)}{\text{Length of genome in base pair} \times 660\text{g/mole} \times 10^{9} ng/g}$$

where, length of *E. coli* genome = 5.2 x 10^6 bp. The standards were run with three technical replicates.

Table 10 *Standard dilution curve*

Prepare standards	H20 (µL)	µL		Genome copies/reaction
S1	407,5	5	from stock	250000
S2	40	10	from S1	50000
S3	40	10	from S2	10000
S4	40	10	from S3	2000
S5	40	10	from S4	400
S6	40	10	from S5	80
S7	40	10	from S6	16
S8	40	10	from S7	3,2

In the 7500 qPCR, the threshold for fluorescence signal was set at 1.0 and analysis was done by 7500 real-time PCR Analysis Software v2.3. In the LightCycler® 96 Instrument, the threshold for fluorescence signal was set at 1.0 and analysis was done by LightCycler® 96 Instrument with LightCycler® 96 SW 1.1 software.

According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). To accept the standard curve for each primer, two requirements must be met. Both the coefficients of determination (R2) are equal to or greater than 0.98. Secondly, the amplification efficiency (E) must be greater than 90%. The R^2 value at greater than 0.98 confirms no errors during pipetting. While the E value at 90% or greater to confirm the correct doubling of targeted DNA during each cycle. The melting curve was checked against the primer melting temperature (Tm) listed by the Sigma-Aldrich product specification to confirm the correct target had been amplified. The E was calculated automatically by the software of both qPCR machines. It is determined by the slope of linear regression with the following formula (Bustin et al., 2009):

$$\text{R efficiency } = 10^{\frac{-1}{slope}} - 1$$

4.2.9 Limit of Detection and Limit of Quantification

Dilutions, including 8 and 1 genomic copies, were also created to investigate and determine the primers' limit of detection and limit of quantification. The limit of detection is the lowest concentration that can be detected and distinguished from a blank sample. The limit of quantification is the lowest concentration that can be reliably detected and meets a predefined cutoff for bias and imprecision (Armbruster & Pry, 2008)

To make 8 genomic copies/µL, 10µL of S6 was diluted with 90µL MB-water. To make 1 genomic copy/µL, 10µL of the 8 genomic copies dilution was further diluted with 70µL of MB-water. For an overview of serial dilutions, see **Figure 5** below.

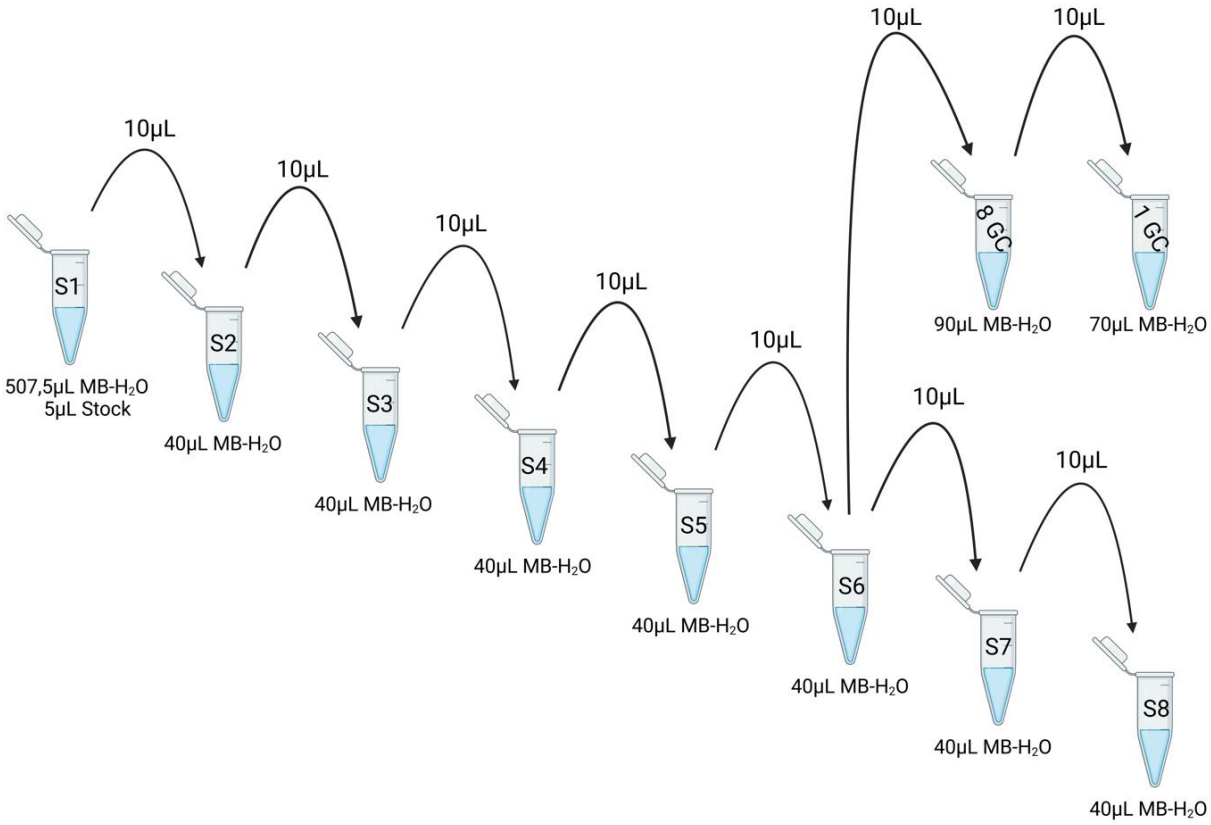


Figure 5 *Serial dilution, from 250 000 to 1 genomic copy Made in Biorender.com*

5. Results

5.1 Comparison of DNA extraction efficiency of three commercial kits

This experiment aimed to evaluate the DNA extraction efficiency of three commercially available kits when using human faecal samples. The kits used were the DNeasy PowerSoil Pro Kit, PureLink™ Microbiome DNA Purification Kit, and QIAamp Fast DNA Stool Mini Kit. Repeated testing and optimisation of each of the DNA extraction kits were performed on multiple aliquots of the same sample to find the one that gives the best results; highest DNA yield within the acceptable purity range. While optimising these kits, eight different process modifications were investigated to develop an optimised DNA extraction protocol. An overview of the different process improvement steps can be seen in **Table 11** below.

Table 11 *Overview of modification that were atempted*

	PowerSoil	PureLink	QIAamp
Explore sample dilution (1:2, 1:3, 1:5).[6]	✔	✔	✔
Explore dilution liquid (MQ-H_2O, 70%EtOH, 95%EtOH).[7]	✔	✔	✔
Use a higher amount of early lysate.[1]	Not possible to test	Not possible to test	✘
Bead beating[3]	✔	✔	✔
Explore different bead-beating settings.[3]	✔	✔	✔
Change from provided bead tubes to third-party bead tubes.[2]	✔	Not tested	Not tested
Explore different lysate and supernatant transfer volumes.[4]	✔	✔	✔
Two-step elution (w/ incubation).[5]	✔	✔	✔

✔ indicate a positive process improvement and ✘ indicate a negative effect in the studied protocol.

All samples used in this study were unmodified raw faecal samples. To perform a more standardised workflow a modification to the protocol in all three kits were established by diluting all samples. Different dilution factors were investigated 1:2, 1:3 and 1:5. The result was that factor 1:3 dilution with MilliQ-water was most effective. This majorly improved sample handling without compromising DNA quality. MilliQ-water was used over MB water during the optimisation process because of availability and because the extracted DNA was not planned to be used later for qPCR. The dilution was also tested using ethanol 95% in 1:3 dilution factor. However,

diluting the samples with 95% ethanol made the sample pellet more difficult to handle. Despite the handling difficulties caused by the ethanol, it improved the quantity and purity of extracted DNA. See **Figure 6** for a comparison of the average DNA yield results with both water and ethanol dilution.

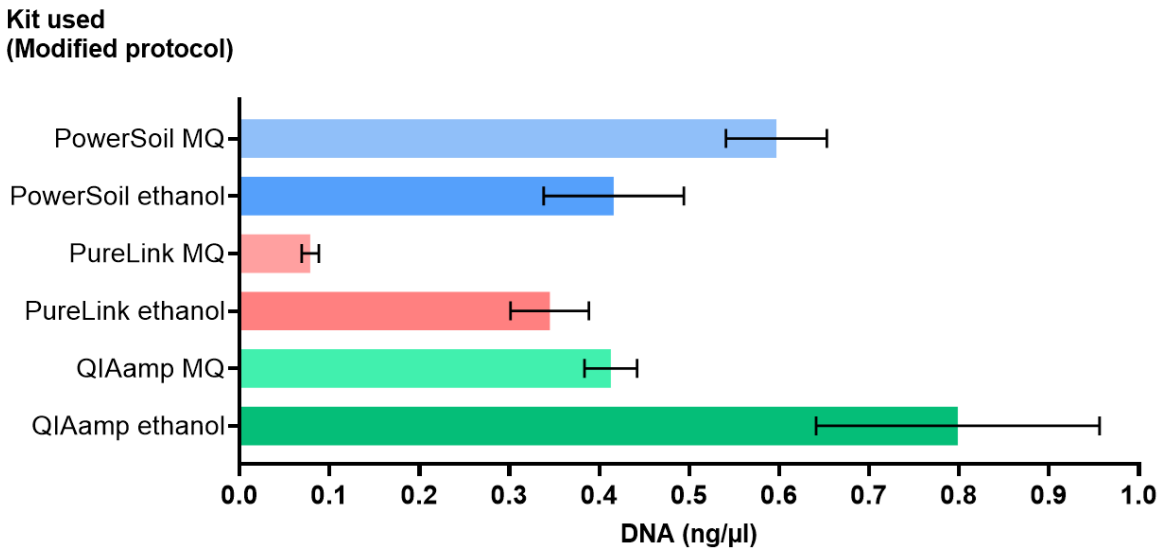


Figure 6 *Comparison of DNA extraction kits Average DNA yield in ng/µL per mg starting material.*

Initially, to improve the total DNA yield using the QIAamp kit, the amount of lysate used was increased from 200µl to 600µl. After chemical- and mechanical cell lysis, there was leftover lysate, and instead of disposing of the extra lysate, 600µl was used in the spin column. The spinning process was repeated three times with 200 µl. The attempt was made to spin the lysate down through the silica filter as extra steps, however, it proved ineffective due to the filter's capacity limits, and the silica filter was not able to withstand the process of repeated spinning.

In the original protocol for the PowerSoil kit, the primary mechanical lysis step involved vortexing the sample. In the modified protocol, a beat-beating step was introduced with the settings of repeated bead-beating two times in 23 seconds at 6500 rpm, with a 30-second pause in between. That proved as a beneficial extra mechanical lysis step in the protocol. Secondly, the bead-beating tube was replaced with a third-party model; Lysing matrix E, to evaluate its influence on the mechanical

lysis step. The combination of the two bead-beating protocol improvements proved to give a positive increase in final DNA yield in the PowerSoil kit and was therefore introduced to the other two kit protocols. This made the best DNA extraction improvements out of all modifications.

Subsequently, when centrifuging the sample during the supernatant transfer step, variable amounts of supernatant were left in the tube depending on the pellet size. Common in the three kits were to provide a volume range for the supernatant leftover (e.g. 500-600µl in the PowerSoil kit). In this modification step, an investigation was conducted to evaluate the optimal supernatant transfer volume, and it was shown that transferring supernatant without any contamination from the pellet was absolutely essential. If remnants of the pellet were passively transferred with the supernatant it negatively affected the DNA purity.

Additionally, to enhance elution, the spin column with the silica filter was incubated twice at 37°C in five minutes with elution liquid before the final centrifugation step. This significantly improved the DNA yield.

Some of the mentioned modifications caused reduced time efficiency by adding extra steps, e.g. diluting, two-step elution and incubated elution. However, these modifications collectively enhanced the yield of DNA extraction. Other steps, like bead beating, improved time efficiency by taking 1 minute for all tubes simultaneously instead of vortexing one by one. The end results of DNA yield and purity after optimising the three kits, including the previously described process improvement steps, can be seen **Table 12**.

Table 12 *Comparrison of DNA extraction kit results*

Kit used	Average µg DNA yield/sample mg	SD µg DNA yield/sample mg	Average A260/A280*	Average A260/A230**
PowerSoil				
MQ-dilute	0,59	0,09	2,03	1,20
EtOH-dilute	0,41	0,13	2,05	1,27
PureLink				
MQ-dilute	0,07	0,01	1,65	0,55
EtOH-dilute	0,34	0,07	1,94	1,15
QIAamp				
MQ-dilute	0,41	0,05	2,07	1,50
EtOH-Dilute	0,79	0,27	2,13	1,83
Baseline***	0,09	n/a	1,92	1,01

*Optimal A260/A280 ratio: ~1.8 is considered “pure” for DNA (Thermo Fisher Scientific, 2015b).
** Optimal A260/A230 ratio: 2.0-2.2 is considered “pure” nucleic acid (Thermo Fisher Scientific, 2015b).

*** For comparison, a baseline run according to the manufacturer's recommendation. n=1.

With all modifications made, QIAamp outperformed the other two kits (PowerSoil, PureLink) not just by its DNA quantity and purity but also by having the option to elute twice as much (µL) from the same starting sample (mg) compared to the other kits without a decrease in DNA concentration (ng/µL) by using more lysate. See **Figure 7** below for the optimised protocol.

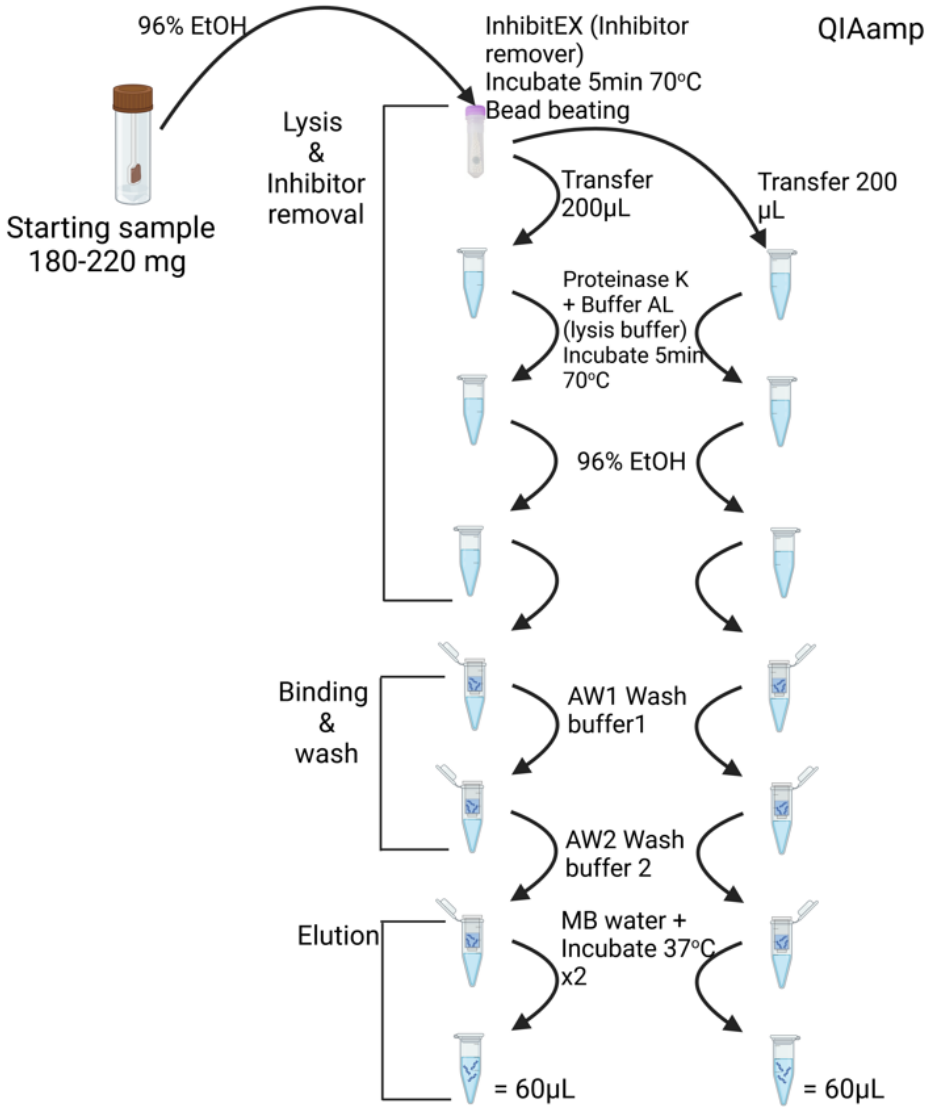


Figure 7 *QIAamp all modified. Enough lysate from bead tube to extract at least 2 two separate procedures. Made in Biorender.com*

5.2 DNA Extraction from stool samples containing low *E. coli* amounts

The established QIAamp protocol was used to extract DNA from stool samples of 5 individuals who were shown by metagenomic sequencing to have low or no detectable *E. coli* levels in the sample (Ken Linstedth unpublished data). The QIAamp protocol was optimised on an infant sample, these 5 samples came from adult volunteers. As adults have a more complex diet compared to infants, it was possible there would be a drop in the A260/A230 ratio from unabsorbed nutrients found in the adult diet. Such as carbohydrates, protein, and lipids not found in infants' diets. The results from extracting DNA can be seen below in **Table 13**. The extracted DNA was used in subsequent testing of primer specificity.

Table 13 *DNA extraction results for stool samples from 5 adults. Samples were lysed, after bead-beating, the lysate was aliquoted before subsequent steps. Bead tube 1 aliquoted to 1,1 and 1,2. Sample ID 51483487 was used twice in two different bead tubes, 1 and 6.*

QIAamp	**Qubit**	**NanoDrop**		
Sample ID (bead tube)	Concentration ng/µL	Concentration ng/µL	A260/A280	A260/A230
51483487 (1,1)	92,6	299	1,88	1,44
51483487 (1,2)	93,2	210	1,94	1,98
51501703 (2,1)	54,6	147,5	1,99	1,63
51501703 (2,2)	70,8	167,8	2,01	1,77
51496715 (3,1)	57,2	107,3	1,97	1,84
51496715 (3,2)	56,4	100,3	1,98	1,86
51496720 (4,1)	87	205,7	1,85	1,29
51496720 (4,2)	89,2	250,2	1,76	0,95
51507773 (5,1)	106	245,7	1,98	1,78
51507773 (5,2)	100	336,3	1,96	1,94
51483487 (6,1)	114	267,9	1,96	2,03
51483487 (6,2)	118	256,3	1,95	2,09

5.3 Determination of the efficiency of primers used for qPCR

First, I tested three different primer pairs gidA, dcp, and U16S, by making a standard curve with each of them. The standard curves were made with technical triplicates of diluted stock of *E. coli* ATCC 25922 genomic DNA, starting at 250,000 genomic copies and 5-fold dilution down to 3,2 genomic copies. There were three sets of curves, one curve for each primer pair. The melting curves showed one product for all primer pairs, indicating that the primers specifically bind to targeted genes (**Figure 8**)

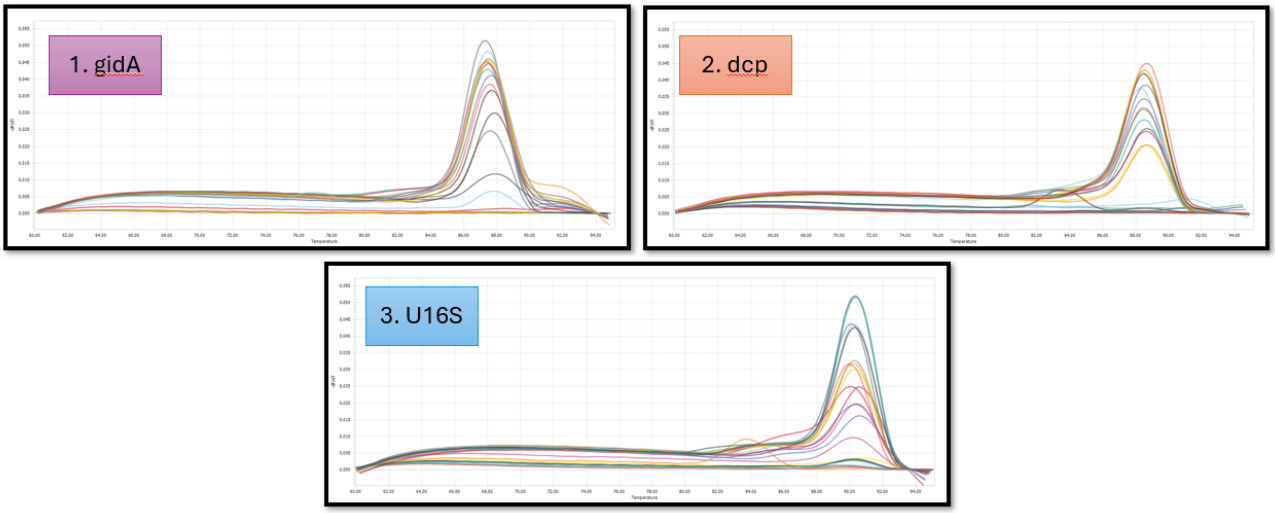


Figure 8 *Melting curves for three primer pairs used**.** Panel 1: The gidA primer pair had a melting peak at an average temperature of 87,52°C. Panel 2: The dcp primer pair had a melting peak at an average temperature of 88,56°C. Panel 3: The U16S primer had a melting peak at an average temperature of 90,22°C.*

5.3.1 Limit of Detection and Limit of Quantification

Since both primer pairs targeted *E. coli dcp and gidA* genes performed equally well, the following steps were done initially only with the *gidA* primer. First, a standard curve was made with 10 replicates for 16, 8, 3,2, and 1 genomic copies. The standard curve and the 10 replicates for limit of detection and limit of quantification were made with technical triplicates of diluted stock *E. coli* ATCC 25922 DNA, starting at 250,000 genomic copies and 5-fold dilution down to 3,2 genomic copies and wells containing 8 and 1 genomic copies. This experiment resulted in unexpected bimodal peaks at 85,5°C and 90°C (**Figure 9**), but the triplicate blank was negative. For 3,2 genomic copies, 8 of 10 wells had a Ct of <40. Mean Ct value (threshold value) of 35,84. For 1 genomic copy, 4 of 10 wells had a Ct of <40. Mean Ct value (threshold value) of 36,99. With these results, we can conclude that the primer behaved unexpectedly, perhaps contamination in the serial dilution as the negative control was negative. The same experiment was run with dcp, however the negative control flagged positive (not shown).

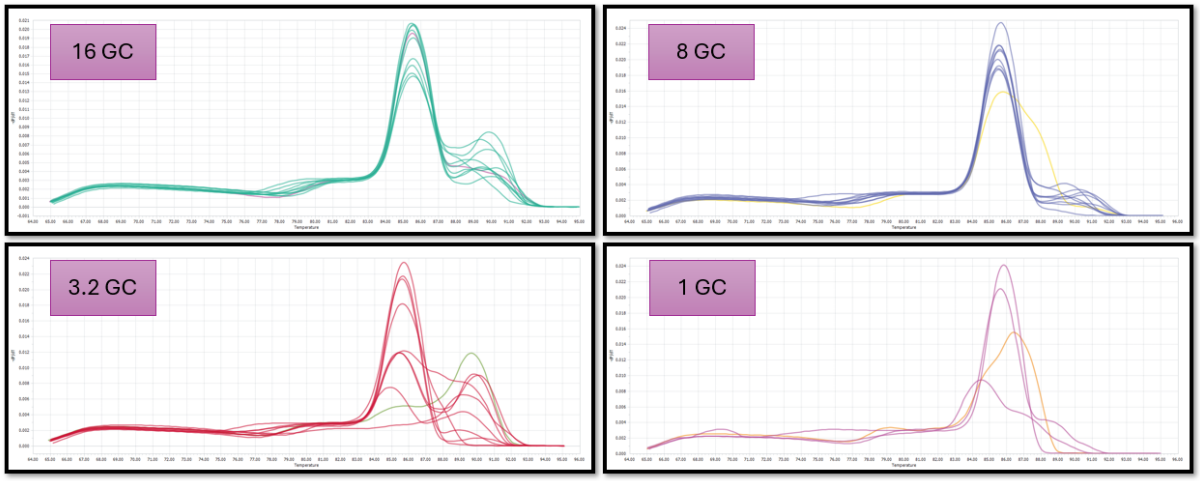


Figure 9 *Limit of Detection and Limit of Quantification with gidA primer. 16, 8, 3,2, and 1 genomic copy*

Since the *gidA* primer pair showed unspecific binding, the dcp primer was also tested in the next step. The same standard curves and the 10 replicates for limit of detection and limit of quantification were made with technical triplicates of diluted stock *E. coli* ATCC 25922 DNA, starting at 250,000 genomic copies and 5-fold dilution down to 3,2 genomic copies and wells containing 8 and 1 genomic copies. The triplicate blank was negative. For 3,2 genomic copies, 8 of 10 wells had an Ct of <40. Mean Ct value (threshold value) of 37,29. For 1 genomic copy, 2 of 10 wells had an Ct of <40. Mean Ct value (threshold value) of 38,21 (**Figure 10**). These results conclude that the dcp primer can detect E. coli down to 3,2 genomic copies.

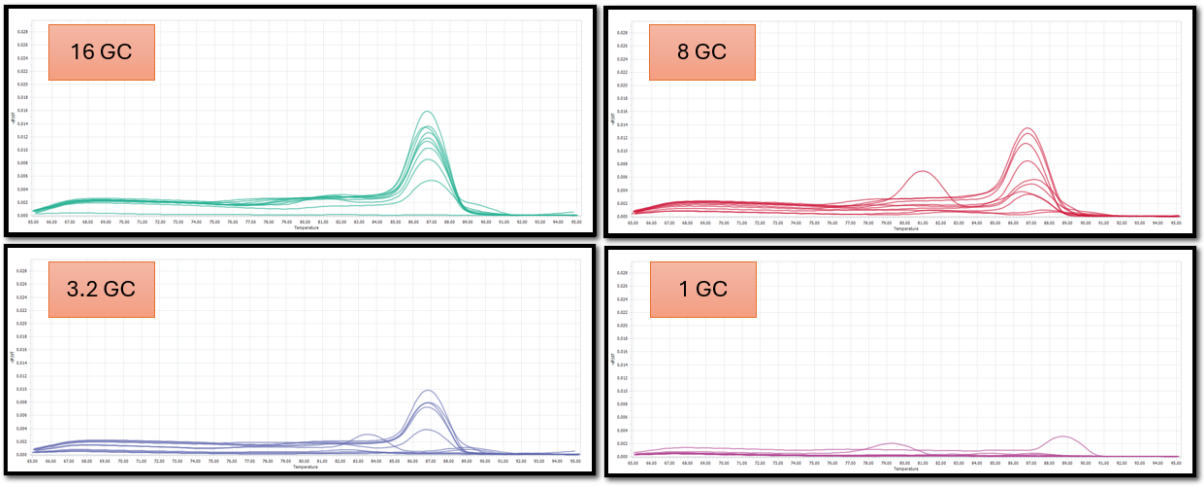


Figure 10 *Limit of Detection and Limit of Quantification with dcp primer. 16, 8, 3,2, and 1 genomic copy*

5.3.2 Test of qPCR protocol on stool samples containing low levels of *E. coli*

In this experiment, seven human stool samples with low or non-detectable abundance of *E. coli* were selected to be tested with *dcp* primer pair. This experiment determined that five of seven samples did not resemble the standard curve (**Figure 11,** panel **I)**. The remaining two out of these seven samples showed melting peaks with a close resemblance with the standard curve, highlighted in panel **II** and shown together with the standard curve in panel **III**. With melting peaks at 87,78 ºC and 86,87 ºC respectively. The standard curve had a melting peak at 86,63 ºC Therefore, these two samples were not used for the following qPCR runs with a pooled sample described below. Samples in this experiment were run as technical quintuplicates.

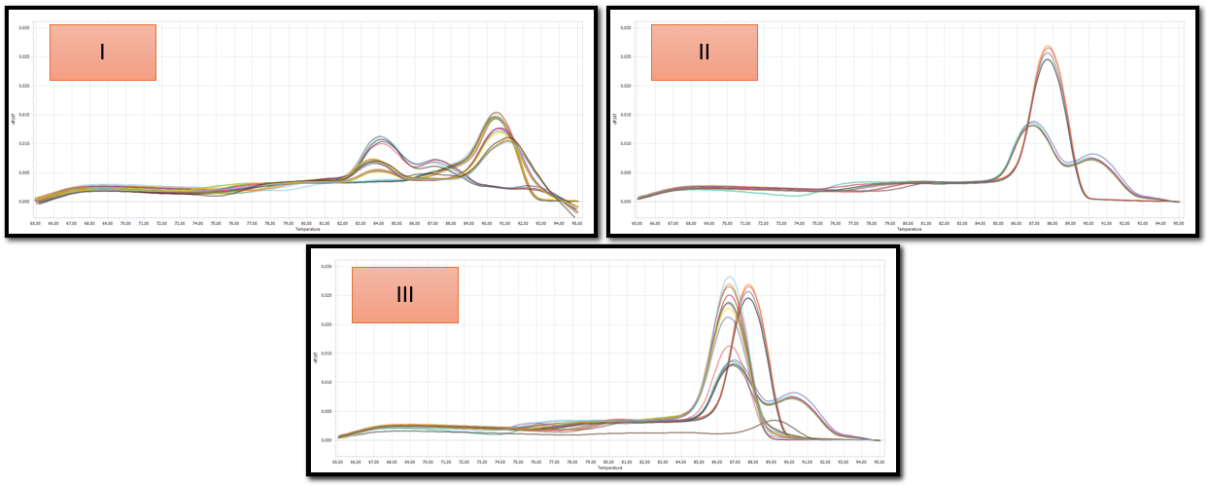


Figure 11 *Seven human faecal samples with dcp primer. Panel I: Five samples without resemblance of the standard curve. Panel II: Two samples with melting peaks closely resembling the standard curve. Panel III: Melting peaks from the standard curve and the two samples resembling the standard curve.*

Because of the previous experiment, the two samples with a resemblance to the standard curve were removed from further testing. The remaining five samples were pooled together. This experiment was to do qPCR using the *dcp* primer with the pooled DNA from the five samples that were determined to have low *E. coli* abundance. To have a direct comparison, a standard curve was set up with the *dcp* primer pair, with technical triplicates of diluted stock *E. coli* ATCC 25922 DNA, starting at 250,000 genomic copies and 5-fold dilution down to 3,2 genomic copies. The pooled DNA had been diluted similarly, starting at 250,000 genomic copies and 5-fold dilution down to 3,2 genomic copies. The standard curve with E. coli ATCC 25922 DNA had a melting peak at 86,60ºC (**Figure 12, panel I**). The pooled samples

dilution curve had a melting peak at 86,51ºC, with a bimodal peak (**Figure 13, panel II**). The second peak had an average melting temperature of 90,56ºC. Because of this peak at 90,56ºC. Because of the unexplainable peaks, it was suspected that the dcp primer was binding to something that's not *E. coli* in the samples. Because of this, it was decided to re-do the human faecel background experiments with the gidA primer.

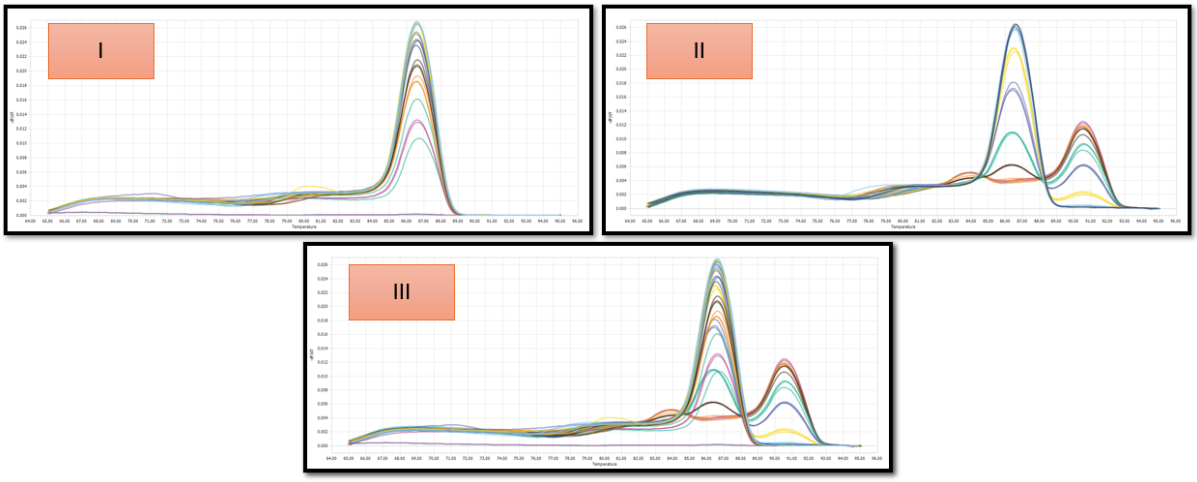


***Figure 12**: Pooled faecal background, dcp Panel I show the standard curve based on genomic E. coli DNA. Panel II shows the serial diluted pooled sample. Panel III shows both the Standard curve and the E. coli sample together.*

Since the *dcp* primer pair showed unspecific binding on the pooled sample, the next experiment involved repeating the qPCR run with the *gidA* primers. In this experiment, the 5 samples used to make a pooled sample in the previous experiment were run again, but this time without being pooled. Two out of five samples had an average melting peak of 86,93ºC, these two samples are shown in **Figure 13**, panel **I**. The remaining 3 samples had an average melting peak of 89,96ºC, as shown in panel **II**. Panel **III** show the same peaks as panel I, but with the standard curve for comparison, the average melting peak temperature of the standard curve was 85,56ºC. The conclusion of this experiment is that the primers are potentially binding to something other than *E. coli*.

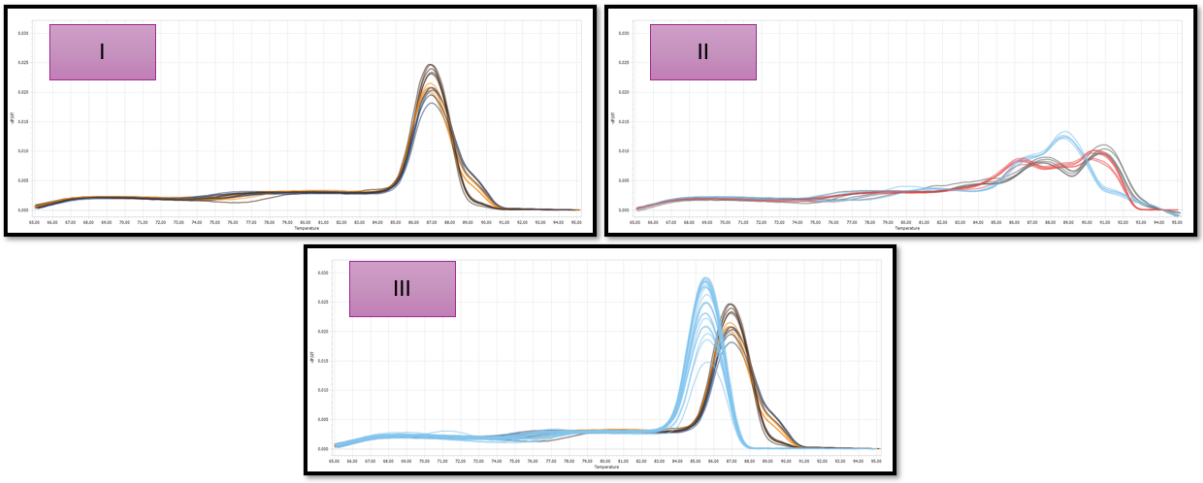


Figure 13 *Human faecal background, five samples, gidA primer. Panel I: Two out of five samples created a peak that resembles the standard curve. Panel II: Three of the samples with no resemblance to the standard curve. Panel III: Standard curve and the two samples in panel I, highlighting their similarity.*

5.3.3 Test of qPCR method on DNA extracted form a *Klebsiella* isolate

A final experiment was conducted because the previous experiments with human faecal samples raised concerns that the *gidA* and *dcp* primers were binding to something other than *E. coli* DNA present in the samples.

Two standard curves were constructed with 5-fold dilution from 250 000 to 3,2 genome copies of *Klebsiella* species complex, one with gidA primers and one with dcp primers. Both primers bind to *Klebsiella* genomic DNA and make nice-looking peaks (**Figure 14)**. The melting curve with *gidA* primers had an average melting peak temperature of 86,88ºC. With the *dcp* primers, the average melting peak temperature was 89,12ºC. In other words, this means the primer does not bind specifically to only *E. coli* DNA, but it also binds to other *Enterobacterales.* In this case, they both bind to *Klebsiella*. As a control, the technical triplicate blanked with 2,5µL MB-water was negative.

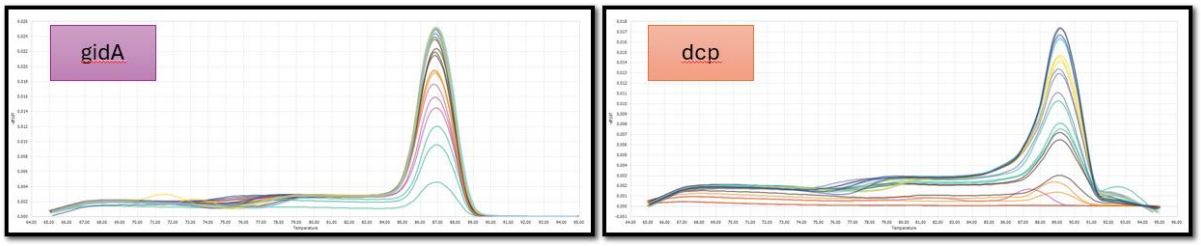


Figure 14 *Klebsiella isolate, Panel gidA shows melting peaks with gidA primer, while panel dcp , shows the melting peaks with dcp primer.*

6. Discussion

6.1 Optimization of DNA Extraction from Human Stool Samples

In this thesis, I attempted to establish a DNA extraction protocol for human faecal samples. I compared three different commercial DNA extraction kits, making several improvements and then comparing them along the way.

The main findings here are that some easy tweaks to the commercial protocols have the potential to increase the quantity of extracted DNA substantially. Here I found three easy improvements that worked well for our samples. Bead beating with a high-powered homogeniser takes little effort and is easy to employ. Commercial kits come standardised in a "fit for all." For the two kits that did include a bead tube, changing it for a tube specialised to work on faecal samples is also beneficial at a low increase in cost required for the project. Besides bead beating, spreading elution over two centrifugations and, at the same time, letting the spin column silica filter fully hydrate before both centrifugations also led to an increased quantity of eluted DNA while making no difference in the final elute volume. The third modification was diluting the sample with either ethanol or water before the addition of the first buffer and bead-beating. Originally, this was used as a way to standardise the volume of samples across experiments but also to reduce the difficulty of handling samples with loops. However, diluting with ethanol increased the quantity and purity of DNA in the final elute.

To optimise the bead beating procedure, several settings were attempted on the Precellys® Evolution Homogenizer. It was found that a more aggressive beating protocol led to increased DNA yield. However, fragmenting the DNA too much and generating heat are concerns that were not explored, but they could be important considerations for downstream applications such as next-generation sequencing and species-specific PCR. In my case, heat generation was not likely to have caused any problems as the lysis buffer that was present in the bead tube together with the sample was also supposed to be heated up and incubated at 70^{o}C; as for the downstream application after DNA extraction, which in my case was qPCR, the primers I used bind to regions that made a short PCR product (164 base pairs for *gidA* and 144 bp for *dcp*). Therefore, I suspect that having some slight fragmentation of DNA would not affect the qPCR result.

Since I had access to the same homogeniser and bead tube as previously described (Lindstedt et al., 2022). I used the same bead-beating settings for this thesis. This would best let me compare our results against each other.

Next, the elution step was modified from using the provided buffer to using MB water. This is because of avoiding possible downstream interference from the EDTA present in the buffer. MB water contains no nuclease, DNAase or RNAse, so it does not degrade the DNA. However, the incubation temperature for better yield should have been explored. Several genomic DNA extraction kits from different manufacturers state to heat up elution buffers to increase yield (New England Biolabs, 2024; Thermo Fisher Scientific, 2024a), Therefore, it is not a surprise that it can also work for spin column-based DNA extraction. In my protocol, the only temperature used for the elution was 37^{o}C when incubating before DNA elution. Compared to manufacturers that use higher temperatures, as well as Boom et al., which described a protocol of 10-minute incubation at 56^{o}C for high elution (Boom et al., 1990). However, time did not allow to explore different temperatures further.

In regard to elution volume, a love volume with a high concentration of DNA elute was deemed desirable. Although the original kit protocols have mentioned to use higher volumes of elution buffer, the elution volume was decided to be a total of 60µL as to concentrate the extracted DNA as much as possible. One question arises: would a higher elution volume lead to more DNA without an increase in other contaminants, increasing the A_{260}/A_{230} ratio at the cost of lower concentration? Before being used in qPCR, DNA elute dilution down to 10ng/µL is necessary, so in the end, it was not sure if aiming for such a high concentration was important after all.

Combined with splitting elution into a repeated set of steps (hydrating, incubating, and centrifuging), one could have explored using 3 × 30µL instead of 2 × 30µL. Here it was not explored to use higher volumes. As previously stated, time was a limiting factor. Lower volumes are unlikely to have increased DNA elution.

It should also be explored if using a buffer, even if it includes small amounts of EDTA, would interfere with the downstream application at unacceptable levels. While it has been shown that small EDTA can inhibit at varying degrees (Huggett et al., 2008). The increased pH from an elution buffer compared to MB-water that I used for elution is likely to yield increased DNA elution without any increase in eluted total

contaminants showing up at the A230 spectre. Also, the increased DNA would require the sample to be further diluted. Therefore, it may not lead to any increased interference effect besides the potential, but not proven, inhibitory effects from the buffer itself. QIAGEN states on their website that buffer ATE, the same elution buffer included in the QIAamp kit, has a pH of 8.3 and 0.1 mM of EDTA. The MB-water I used has a pH of 6,3.

As the elute would have to be diluted down to DNA levels of 10 ng/µL before downstream qPCR use, a buffer containing small amounts of EDTA would unlikely cause problems for qPCR. In Lindstedt et al 2022, while using a modified PureLink protocol for DNA extraction, the included S6 elution buffer was used without any mentioned issues (Lindstedt et al., 2022). In Haugan 2018, the included buffer is also used from a QIAamp DNA Mini Kit. This DNA extraction kit includes an elution buffer which includes 0,5 mM EDTA. Haugan reports problems purifying enough bacterial DNA from spleen and kidney tissue, but no issues with amplification of peritoneal lavage fluid, or blood was reported (Haugan et al., 2018).

Finally, for the initial dilution step, the original idea came from the wish to dispense an equal amount of sample when running a comparison. And because up to 30% of the faecal sample got lost in the transfer from one tube to the next when using the loops. With 1:3 (mg:µL) dilution with water, it was easy to pipette the exact amount, and no need to use loops. However, stool samples are often stored in ethanol, which is used as a stabilisation solution during the collection. Therefore, it was also attempted to dilute samples with ethanol. While ethanol dilution did not aid with ease of handling like water dilution did, diluting with ethanol caused the faecal sample to pellet and become brittle and, therefore, was more difficult to handle initially.

However, it surprisingly also increases the quantity of DNA in the final elute, but only for two of the three kits. In the PowerSoil kit, it resulted in a loss of performance (**Figure X Chapter Y**). As this effect was not found with the PureLink and QIAamp kits, and all three kits use patented buffers, it is uncertain as to why this effect happened.

6.2 Development of qPCR for quantitative detection of *E. coli* in stool samples

In this master's thesis, I have attempted to quantify *E. coli* from faecal samples using qPCR. Unfortunately, the outcome did not meet the initial expectations. This was because the primers used in this work did not bind specifically to only *E. coli*. Instead, it was also found to bind strongly to *Klebsiella*.

Primers were chosen based on a publication by Haugan et al. that described the quantification of *E. coli* in a mouse model (Haugan et al., 2018). In hindsight, this was problematic. First of all, as can be seen in the result section, both primer sets for *E. coli* did not bind to only *E. coli* but also the Klebsiella complex that had previously been isolated. The primers likely acted more as general *Enterobacterales* primers rather than *E. coli*-specific primers. The genes targeted by the primers are encoding for two genes located right next to the origin of replication and terminus region of the *E. coli* ATC 25922, and it can be expected that the gene sequences of these housekeeping proteins are conserved across several *Enterobacterales* species (Haugan et al., 2018).

Haugan et al. investigated bacterial growth using a mouse peritonitis model, where *E. coli* was injected into the peritoneum. As the mice developed peritonitis, samples were taken from peritoneal lavage fluid, blood, spleen, and kidneys. Therefore, no other *Enterobacerales* were expected to be found.

Besides the later proven fact that the primers were not specific for *E. coli*, several possible contamination sources were also considered during the qPCR protocol development. For example, the T4 Gene 32 Protein used during the qPCR reaction is produced by an overexpression for the T4 gene from a plasmid in a strain of *E. coli*. Therefore, initially, it was considered that there could be some leftover *E. coli* DNA in the chemical. However, as the negative control was blanked with MB-water and did not show any signs of amplification, it was clear that no leftover DNA was present in this case.

As with the T4 Gene 32 protein, the SYBR could have been a source of contamination. Unlike the T4 Gene 32 protein, the manufacturer does not state how the SYBR is sourced. Either way, as the negative control did not show any signs of amplification, the possibility of SYBR being a source of contamination was also ruled out.

7. Conclusions and Future Perspectives

In conclusion, three DNA extraction kits were tested and modified to improve performance when extracting bacterial DNA from human faecal samples. The optimised QIAamp Fast DNA Stool Mini Kit performed the best of these. With over 8 times as much DNA extracted compared to manufacturers' guidelines and increased purity, it can be considered a huge success.

However, the qPCR part of this thesis was less successful. This thesis hypothesised that it would be possible to quantify the amount of *E. coli* in stool samples. This proved to be more difficult than expected. For a future project, it would be beneficial to spend more time with bioinformatic tools to aid with choosing or designing primers that will do the job for its application. In the case of the *gidA* and *dcp* primers, they should work just fine for applications of quantifying *Enterobacterales* in general.

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9. Appendix

DNeasy PowerSoil Pro Kit

The kit procedure includes both mechanical and chemical cell-lysing. The kit includes a 2mL screw top tube filled with zirconium beads to be vortexed for 10 minutes, along with the chemical lysing buffer, CD1. Lysate then undergoes removal of inhibitors with wash buffer Solution CD2. Transferring supernatant to a spin column silica filter and binding the DNA to the filter with binding buffer CD3, a high salt concentrate solution. The DNA bound to the silica filter is washed in a two-step regime, using solution EA and C5. Solution C5 is an ethanol-based wash solution. The protocol uses a tromethamine-buffered saline (TRIS) based elution buffer for the final elution of DNA. Total elution volume 50-100µL.

QIAamp Fast DNA Stool Mini Kit

The original kit procedure does not include any mechanical lysing step but does include chemical lysing and thermal lysing. The sample and its lysis buffer InhibitEX are incubated at 70ºC for 5 minutes. After centrifugation, the supernatant is transferred to a new tube and mixed with proteinase K and buffer AL. The tube is then incubated again at 70ºC for 10 minutes. Addition of 96% ethanol before transferring and passing the lysate to a spin-column with a silica membrane filter. The ethanol causes the DNA to aggregate and bind to the silica filter. The membrane is then washed in a two-step regime with two wash buffers, AW1 and AW2. The protocol uses a TRIS-based elution buffer for the final elution of DNA. Total elution volume 200µL.

The kit protocol calls for incubating at 95ºC instead of 70ºC for cells that are difficult to lyse, i.e., gram-positive bacteria.

PureLink™ Microbiome DNA Purification Kit

The original kit procedure does not include any mechanical lysing step but does include chemical lysing and thermal lysing. The sample, its lysis buffer S1, and lysing enchanter are added to a bead tube and incubated at 65ºC for 10 minutes. After incubation, the sample is vortexed before being centrifuged, transferred to a new tube, and mixed with a clean-up buffer S3 to remove inhibitors. The lysate is then added to a spin-column with a silica membrane filter and binding buffer S4 to bind DNA to the silica filter membrane. The membrane is then washed with a wash

buffer S5 before being centrifuged twice to remove excess S5 residues that can interfere with downstream applications. The protocol uses a TRIS-based elution buffer S6 for the final elution of DNA. Total elution volume 50-200µL.