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Diagnostics and clinical significance of *Faecalibacterium prausnitzii*

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PREFACE

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Abstract:

The human body contains over ten times more microbial cells than human cells, and over 100 times more microbial genes than human genes. All the microbes (bacteria, archaea and eukarya) residing in and on the human body are together referred to as the human microbiota. The microbial density is largest in the gastrointestinal tract and especially in the colon, where most of the residing microbes are anaerobic bacteria.

Gut bacteria have many important tasks. They, for instance, protect against invading pathogens, participate in food digestion and take part in immune functions. Gut microbiota dysbiosis has been linked to various disorders such as obesity, asthma and inflammatory bowel disease (IBD). *Faecalibacterium prausnitzii* (*F. prausnitzii*) is one of the most abundant bacteria in a healthy human gut, and recent studies suggest this bacterium could be beneficial for gut health. The number of *F. prausnitzii* is significantly decreased in patients suffering from IBD.

The aim of this study was to set up a practical method for quantifying *F. prausnitzii* from fecal samples, and to cultivate this obligatory anaerobe to enable antibiotic resistance testing. Two deoxyribonucleic acid (DNA) extraction protocols were compared to reveal the most suitable method for extracting *F. prausnitzii* DNA from feces. Quantitative polymerase chain reaction (qPCR) was compared with fluorescence *in situ* hybridization (FISH) to discover which method was more practical for studying *F. prausnitzii* abundance from fecal samples.

GXT Stool Extraction Kit was found most effective and user-friendly method for extracting *F. prausnitzii* DNA from fecal samples. Best DNA quantity was gained when combining GXT Stool Extraction Kit with an extra homogenization with MO BIO homogenizer. qPCR with LightCycler automate was found to be the most useful method for analyzing *F. prausnitzii* abundance from feces. Fluorescence *in situ* hybridization (FISH) combined with fluorescence microscopy analysis was found extremely laborious and time-consuming when compared to qPCR. Because *F. prausnitzii* is an obligatory anaerobe with very strict growth requirements, the cultivation of the bacterium was challenging and the antibiotic resistance analysis could not be performed during the time scheduled for this study.

In a small sample cohort (*n* = 27) included in this study, each of the fecal samples contained *F. prausnitzii*. The abundance of this particular bacterium in feces varied substantially between the subjects. The analysis of possible interrelationships between *F. prausnitzii* abundance and the clinical characteristics of the study subjects was excluded from this study due to small sample number. However, this preliminary study provides tools for quantifying the bacterium and assessing its clinical significance in future research.

Tiivistelmä:

Ihmiskeho sisältää yli kymmenen kertaa enemmän mikrobisoluja kuin ihmissoluja, ja yli sata kertaa enemmän mikrobiperäisiä geenejä kuin ihmisperäisiä geenejä. Kaikkia ihmiskehon sisällä ja pinnalla eläviä mikrobeja kuvataan yhteisnimityksellä normaalimikrobisto. Eniten mikrobeja esiintyy ruuansulatuskanavassa ja erityisesti paksusuolessa. Suoliston mikrobeista suurin osa on anaerobibakteereja.

Suolistobakteereilla on tärkeä merkitys ihmisen terveydelle. Bakteerit muun muassa suojaavat patogeenejä vastaan, auttavat ruuansulatuksessa ja osallistuvat immuunipuolustuksen kehitykseen ja toimintaan. Suolistomikrobiston epätasapaino on liitetty moniin sairauksiin ja elimistön toimintahäiriöihin, kuten lihavuuteen, astmaan ja tulehduksellisiin suolistosairauksiin. *Faecalibacterium prausnitzii* (*F. prausnitzii*) on yksi terveen suoliston runsaslukuisimmista bakteereista, ja kyseisen bakteerin esiintyvyydellä uskotaan olevan yhteys suoliston hyvinvointiin. Tulehduksellisista suolistosairauksista kärsivien ihmisten suoliston *F. prausnitzii* –määrän on todettu olevan huomattavasti alentunut.

Tämän tutkimuksen tavoitteena oli pystyttää UTULab-laboratorioon toimiva ja mahdollisimman helppokäyttöinen kvantitatiivinen menetelmä *F. prausnitzii*n diagnostiikkaa varten, jotta tulevaisuudessa kyseisen bakteerin esiintyvyyden tutkiminen ulostenäytteistä olisi mahdollisimman yksinkertaista. Lisäksi tutkimuksen tavoitteena oli saada *F. prausnitzii* kasvatettua ja tutkia bakteerin antibioottiherkkyyttä. Tutkimuksessa testattiin erilaisia deoksiribonukleiinihapon (DNA) eristysmenetelmiä mahdollisimman tehokkaan menetelmän löytämiseksi, ja vertailtiin polymeraasiketjureaktion (qPCR) ja fluoresenssi *in situ* hybridisaation (FISH) soveltuvuutta kvantitatiiviseen *F. prausnitzii* –diagnostiikkaan.

GXT Stool Extraction Kit todettiin toimivimmaksi ja käyttäjäystävällisimmäksi menetelmäksi ulosteen sisältämän *F. prausnitzii* –DNA:n eristyksessä. Paras DNA-pitoisuus saatiin, kun GXT Stool Extraction Kit –menetelmään liitettiin ylimääräinen homogenisointi MO BIO –homogenisaattorin kanssa. qPCR LightCycler –automaatilla todettiin parhaaksi menetelmäksi *F. prausnitzii*n esiintyvyyden tutkimiseen ulostenäytteistä. FISH fluoresenssimikroskooppianalyysillä todettiin qPCR:ään verrattuna todella työlääksi ja aikaavieväksi menetelmäksi. Koska *F. prausnitzii* on ehdoton anaerobibakteeri jonka kasvuvaatimukset ovat erittäin tiukat, bakteerin viljely oli todella haastavaa ja hidasta, eikä antibioottiherkkyysmäärityksiä onnistuttu suorittamaan tutkimusta varten suunnitellun ajan sisällä.

Kaikki tämän tutkimuksen aikana analysoidut ulostenäytteet (*n* = 27) sisälsivät *F. prausnitzii* –bakteeria, mutta bakteerin määrä vaihteli huomattavasti näytteiden välillä. Bakteerin määrän ja tutkimukseen osallistuneiden henkilöiden kliinisten tietojen välisiä mahdollisia riippuvuussuhteita ei pienen otoskoon takia analysoitu. Tutkimuksessa kehitettyjä menetelmiä voidaan kuitenkin tulevaisuudessa hyödyntää *F. prausnitziin* kliinisen merkityksen analysoinnissa.

Avainsanat: Suolistomikrobisto, *Faecalibacterium prausnitzii*, DNA-eristys, qPCR, FISH

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ABBREVIATIONS

AGE	Agarose Gel Electrophoresis
BB	Bead-Beating
Bp	Base pairs
CD	Crohn's Disease
C-section	Caesarean section
C.V.	Coefficient of Variation
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
FAA	Fastidious Anaerobe Agar
FAB	Fastidious Anaerobe Broth
FISH	Fluorescence *in situ* Hybridization
F. prausnitzii	*Faecalibacterium prausnitzii (Fusobacterium prausnitzii)*
GI	Gastrointestinal
GXT	GXT Stool Extraction Kit (Hain Lifescience GmbH, Germany)
IBD	Inflammatory Bowel Disease
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
QIA	QIAamp DNA Stool Mini Kit (Qiagen, UK)
rRNA	Ribosomal Ribonucleic Acid
qPCR	Quantitative Real Time Polymerase Chain Reaction
S.D.	Standard Deviation
UC	Ulcerative Colitis

1. INTRODUCTION

Human microbiota, previously called microflora or normal flora, refers to all the microbes residing in and on the human body (for review see Sekirov et al., 2010). Microbes have colonized virtually every surface of the body that is exposed to the exterior, and for example skin, mouth and gastrointestinal (GI) tract are crowded with microbes.

Human microbiota consists of bacteria, archaea and eukarya (mainly yeasts) that all have an ability to colonize and multiply under certain conditions peculiar to particular body sites. The composition of microbiota varies greatly between different body areas, and each accessible area of the body can be considered as a particular ecological niche (for review see Clemente et al., 2012). In some references, also viruses are listed as members of human microbiota. A huge number of viruses are present in and on the human body, but the role of these virus populations is still somewhat unknown (Minot et al., 2011).

It is estimated that the human microbiota includes about 10^{14} bacterial cells per individual (for review see Sekirov et al., 2010). This number is ten times greater than the number of human cells in a human body. The gene catalog of the microbiota, referred to as microbiome, has also been found to be much more extensive than the human genome (Qin et al., 2010).

The composition of the microbiota of healthy human individuals is extremely complicated. Studies have revealed that especially oral and intestinal communities are very diverse (The Human Microbiome Project Consortium, 2012), but according to a study by Costello and co-workers (2009), several skin locations may have even more diverse bacterial communities than gut or mouth.

The human body has evolved a symbiotic relationship with its microbiota and, for example, requires the colonization of beneficial microorganisms for proper immune development and function (for review see Bäckhed et al., 2005). Thus it would actually be appropriate to think our bodies as compositions of human and microbial cells and genes.

1.1. Gut Microbiota

The human GI tract, also known as the digestive tract, generally refers to stomach and the intestines (Nienstedt et al., 2004). In some references, all the digestive structures from mouth to rectum are included in the definition. The large intestine starts at the ileocecal junction and consists of cecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum (Figure 1) (for review see Gibson and Roberfroid, 1994). Large intestine has an important role in the processing of non-absorbed food material passed from the small intestine (Nienstedt et al., 2004). In addition, it participates in absorption and secretion of certain electrolytes and water and in storage and excretion of waste materials.
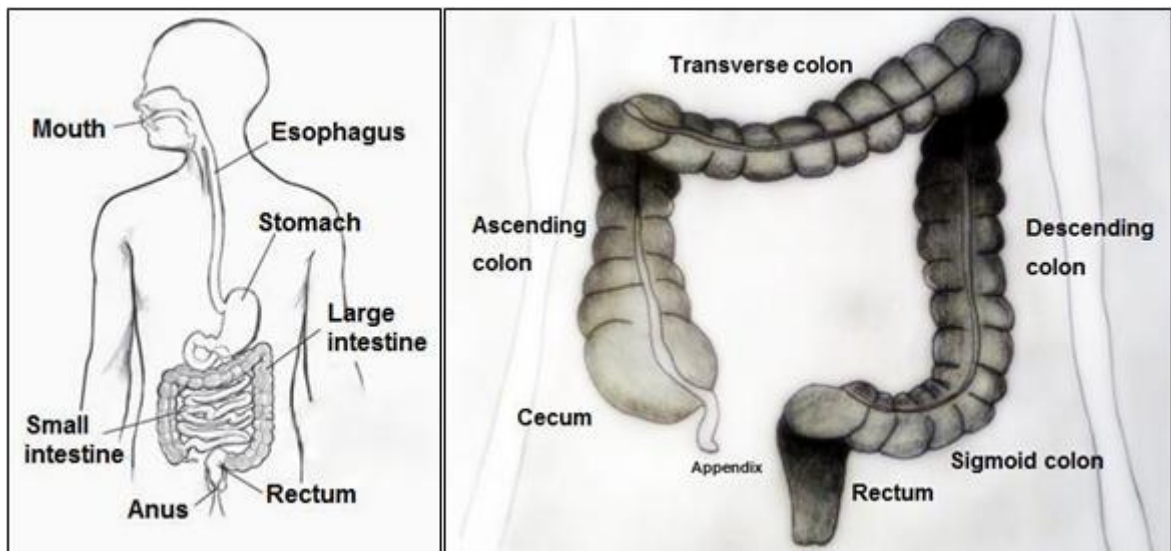
Figure 1: Large intestine as a part of the human GI tract.

The gut microbiota refers to the community of microbes present in the GI tract. Gut microbiota consists of bacteria, archaea (at least one phylotype) and eukarya (for review see Sekirov et al., 2010). GI tract is the major surface for microbial colonization in the human body, and the colon alone is estimated to contain over 70 % of the microbes in the whole body. As a matter of fact, the gut microbiota represents the highest cell density ever recorded for any ecosystem (Whitman et al., 1998).

The gut microbiota is not homogenous (for review see Sekirov et al., 2010). The number of bacterial cells increases dramatically from stomach to colon, and the bacterial phyla and species composition vary greatly between the different parts of the gut (Figure 2). In

addition to the longitudinal heterogeneity, also latitudinal variation has been stated; the microbial composition of the intestinal lumen differs significantly from the microbes attached to or embedded in the mucus layer.
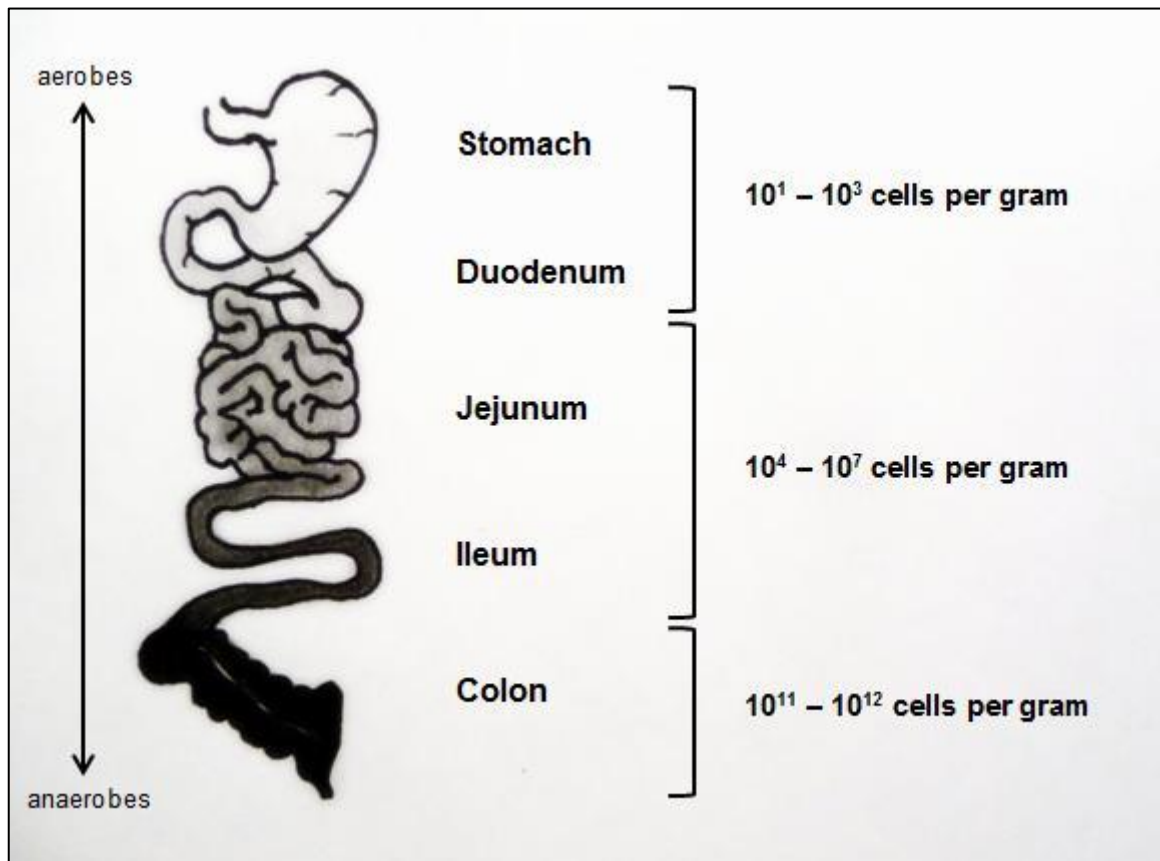
Figure 2: Bacterial concentrations across the length of the GI tract. Concentrations are presented as numbers of bacterial cells per gram of the intestinal content. Based on Sekirov et al., 2010.

The genetic catalog of all microbial species within the GI tract is referred to as gut microbiome. Gut microbiome is claimed to contain over 100 times more genes than the human genome, and it provides some genetic and metabolic attributes that the human host has thus not been required to evolve (Qin et al., 2010).

Most of the gut microbes are strictly anaerobic bacteria. Phyla *Bacteroidetes* (especially genus *Bacteroides*) and *Firmicutes* (especially genus *Faecalibacterium*) are confirmed to be the most abundant members residing in the human gut (Eckburg et al., 2005). *Actinobacteria*, especially genus *Bifidobacterium*, are also relatively common, but not equally dominant members of the gut microbiota (Arumugam et al., 2011). In a recent investigation by Arumugam and co-workers (2011), it has been suggested that the gut

microbiota of most individuals could be categorized into enterotypes based on the dominant bacterial genera (*Bacteroides*, *Prevotella*, or *Ruminococcus*) present in the GI tract. Enterotype clustering seems to be mostly affected by long-term dietary habits and is independent of nationality, age, sex and body mass index (Arumugam et al., 2011; Wu et al., 2011).

1.1.1. The development of gut microbiota

The human microbiota is acquired during and right after birth, and undergoes dynamic changes during development (Penders et al., 2006; Palmer et al., 2007). The mode of delivery, type of feeding (breastfeeding or formula-feeding) and antibiotic use are the most important factors affecting the gut microbiota development of infants. The gut microbes of newborns vary greatly between individuals and in the course of time, but the intestinal microbiota of a one-year-old infant has, to some extent, already stabilized and converged towards the profile characteristic of an adult GI tract microbiota.

Babies are born essentially sterile, and receive bacteria from the environment immediately upon birth (Dominguez-Bello et al., 2010). Vaginally delivered infants receive their first bacteria as they pass through the birth canal and thus have microbial communities resembling those found in the vaginal microbiota of their mothers, dominated by *Lactobacillus*, *Prevotella* or *Sneathia* spp. Babies born by Caesarean section (C-section) don't receive vaginal microbes but instead get their first bacteria usually from the skin microbiota of their mother, dominated by taxa such as *Staphylococcus*, *Corynebacterium* and *Propionibacterium* spp.

There is increasing evidence that the early colonization of bacteria affects the health of the infants and also influences the host health later in life (for review see Johnson and Versalovic, 2012). The delivery mode may influence the immunological functions during the first year of life by affecting the development of gut microbiota. Babies delivered by C-section are found to have significantly lower bacterial cell counts in fecal samples and are shown to develop stronger non-specific humoral immune responses (Huurre et al., 2008). C-section infants seem to also have a higher risk for some immune-mediated diseases like asthma later in life (van Nimwegen et al., 2011). Babies born by C-section are also found

to be colonized by *Clostridium difficile* more often than vaginally born infants (van Nimwegen et al., 2011; Penders et al., 2006).

It has been noted that *Faecalibacterium prausnitzii* (*F. prausnitzii*) is not detectable in the fecal samples of babies under 6 months of age (Hopkins et al., 2005.). After that, the number starts to increase gradually, and children of 1 to 2 years of age already have a significant amount of *F. prausnitzii* in their GI tract.

1.1.2. Factors determining the gut microbiota composition

Gut microbiota is dynamic, and the microbes undergo continuous selective pressure (for review see Lozupone et al., 2012). Therefore gut microbiota changes over time and each individual appears to have a unique gut microbiota composition. Age, genetics, environment and dietary habits are factors causing baseline variations in the gut microbiota composition between individuals. Factors that can alter the composition of gut microbiota of an individual during time are, for example, dietary changes, drugs (especially antibiotics), stress and changes in the physiological state of the host.

1.1.2.1. Baseline variations

Twins and mother-daughter pairs are found to have more similar gut microbiotas than two unrelated individuals (Turnbaugh et al., 2009). This suggests that there may be a genetic influence on the microbiota composition, but this can at least partly be explained by the environmental factors (Dicksved et al., 2008). Both age- and gender-related differences in fecal microbiota have been observed (Mueller et al., 2006). It has been found that the number of enterobacteria in feces is higher in elderly than in working-age people, and the levels of *Bacteroides-Prevotella* group seem to be higher in males than in females. Variation in gut microbiota composition between individuals from different countries has also been observed (Nam et al., 2011). These regional differences are thought to be mainly due to dietary habits and genetics.

1.1.2.2. Antibiotics

One of the major factors perturbing the composition of human gut microbiota is the use of antibiotics (for review see Clemente et al., 2012). Antibiotics can alter the microbial

composition of the gut dramatically; in infants, the microbial density of the GI tract can decrease strikingly due to antibiotics (Palmer et al., 2007), and antibiotic treatment has been shown to result in the reduction of the total bacterial number also in adult fecal samples (Bartosch et al., 2004; Swidsinski et al., 2008).

Antibiotic-induced changes in the gut microbiota are usually temporary, but long-term microbial population fluctuations have also been reported (for review see Preidis and Versalovic, 2009). Antibiotics may even move the gut microbiota to a new, alternative stable state. Antibiotic-induced alterations in the gut microbiota raise the disease risk by increasing the susceptibility to pathogen colonization; for example, diarrhea caused by *Clostridium difficile* is a well-known consequence of antibiotic courses.

1.1.2.3. Dietary changes

Changes in diet can induce significant changes in the gut microbiota (for review see Flint, 2012). It is, for instance, known that the dietary intake of indigestible carbohydrates affects the number of bacteria in the colon and influences microbial fermentation. Some examples of diets that are found to affect the gut microbiota are carbohydrate- or fat-restricted low calorie diets (Ley et al., 2006) and fiber-restricted diets (Benus et al., 2010).

Obese people are known to have lower microbial diversity in their gut than lean people (Turnbaugh et al., 2009). The abundance of specific taxa and functional genes are also significantly different between obese and lean individuals. Actually, it has been claimed that people could be classified as lean or obese with 90 % accuracy simply by the gut microbiota composition (for review see Lozupone et al., 2012).

1.1.2.4. Prebiotics and probiotics

Prebiotics and probiotics have been widely used to shape the gut microbiota (for review see Huebner and Surawicz, 2006). Prebiotics are indigestible food ingredients that have a beneficial effect on the intestinal tract through their selective metabolism (Gibson and Roberfroid, 1994). Three criteria have been given to prebiotics: They need to be resistant to gastric acidity, hydrolysis by mammalian enzymes and to gastrointestinal absorption,

they must be fermented by gut bacteria and they must selectively stimulate the growth and/or activity of the health advantageous intestinal bacteria (Gibson et al., 2004).

Fuller (1989) has described probiotics as live microbial feed supplements that improve the intestinal microbial balance and thus affect the host beneficially. More recently, probiotics have been defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). In other words, both pre- and probiotics provide dietary means to support the balance of GI tract microbiota.

The possibilities of using pre- and probiotics are under constant research (for review see Huebner and Surawicz, 2006). The aim of many research groups is to develop products that can improve health through manipulation of the gut microbiota. This is a challenge, because the effects of pre- and probiotics often seem to be contradictory; some people clearly benefit from the products while others do not benefit at all (Reid et al., 2010). The differences in responding to pre- and probiotics may arise from the differences in the gut microbiota composition between individuals.

1.1.3. The significance of gut microbiota

Most of the microbes inhabiting the gut are either harmless or beneficial to host. These commensal and symbiotic bacteria have many important tasks. They, for instance, protect the host against invading pathogens (for review see Chow et al., 2010), participate in food digestion (Bäckhed et al., 2004), and take part in immune functions (for review see Fung et al., 2012). In recent research, gut microbiota imbalances are linked to very diverse disorders. Disturbances in the balance between gut microbiota and the host have been associated, among many others, with obesity (Ley et al., 2005), type 2 diabetes (Bäckhed et al., 2004), inflammatory bowel disease (Noor et al., 2010; Manichanh et al., 2006) and neurological disorders like autism (Williams et al., 2012). Gut microbiota dysbiosis may also have a role in cancer development (For review, see Sekirov et al., 2010) and immunological disorders like allergies (for review see Round & Mazmanian, 2009). Gut microbiota may even influence the brain development and behavior (Collins and Bercik, 2009; Heijtz et al., 2011).

1.1.3.1. Impact on food digestion

Gut microbiota is able to utilize otherwise inaccessible nutrients from our diet (for review see Bäckhed et al., 2005 and Sekirov et al., 2010). For example, gut bacteria are known to process otherwise indigestible dietary polysaccharides, such as plant-derived pectin, cellulose, hemicellulose and resistant starches. In addition, gut microbiota has an ability to produce essential vitamins like vitamin K and vitamin B_{12}. Studies with germ-free mice suggest that gut microbiota strongly affects the energy harvest from the diet by promoting the absorption of monosaccharides from the gut lumen (Turnbaugh et al., 2006). Gut microbiota also seems to affect the energy storage of the host by inducing hepatic lipogenesis (Bäckhed et al., 2004).

1.1.3.2. Impact on disease development and immune functions

The main purpose of the immune system is to protect the human body from infections (Nienstedt et al., 2004). The immune system is in charge of recognizing and either responding or adapting to numerous signals sent by foreign and self-molecules. Healthy steady state in the human gut requires constant interactions and delicate balance between the human host and the gut microbiota (for review see Round and Mazmanian, 2009). The human body has evolved various physical and molecular mechanisms for maintaining homeostasis with commensal microorganisms, and commensal bacteria have developed several mechanisms to crosstalk with human cells that play key roles in maintaining homeostasis.

Gut microbiota, alongside with the host factors, prevents colonization of the invading pathogens in the GI tract (for review see Chow et al., 2010). This important natural defense mechanism against opportunistic pathogens is called colonization resistance. If the balance of the gut is disturbed, it can lead to disruption of colonization resistance which often leads to inflammation or disease. In addition to maintaining colonization resistance, gut microbiota is also proposed to provide other immunological benefits to the host; it has been recognized in germ-free mouse experiments that the gut microbiota influences the development of normal mucosal immune system and thus unfavorable alterations in the gut microbiota may lead to immune dysfunction and/or autoimmunity (for review see Fung et al., 2012).

The impact of gut microbiota on gut health has been exemplified in studies of inflammatory bowel disease (IBD). IBD is a chronic, relapsing inflammatory disease characterized by mucosal damage in genetically susceptible hosts (Baumgart and Carding, 2007). Two main types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). The incidence of IBD has been persistently increasing worldwide and has been linked to the lifestyle of developed countries (for review see Manichanh et al., 2012).

CD is a chronic inflammatory disorder mainly affecting the GI tract, and its common symptoms are pain, fever, bowel obstruction and bloody diarrhea (for review, see Sartor, 2008). CD is thought to be caused by interactions between environmental, immunological and bacterial factors in genetically susceptible individuals, and the disease symptoms are suggested to occur due to the imbalanced immune response against the commensal gut bacteria (Baumgart and Sandborn, 2012). A significant decrease of certain bacteria in CD patients has been reported in several publications (Manichanh et al., 2006; Sokol et al., 2009; Dicksved et al., 2008), and the abundance of enterobacteria seems to be significantly higher in CD patients than in healthy subjects (Seksik et al., 2003).

UC is a chronic disease characterized by obscure mucosal inflammation in the colon (for review see Langan et al., 2007). The precise etiology of UC is still unclear, yet some genetic components are presumed to increase the disease susceptibility (Baumgart and Carding, 2007). Distinct microbiota abnormalities are stated in patients with UC (Frank et al., 2007; Noor et al., 2010).

Because dysbiosis and decreased complexity of the gut microbiota is common in IBD patients, the exact role of gut microbiota in IBD development is under constant research (for review see Manichanh et al., 2012).

1.2. *Faecalibacterium prausnitzii*

F. prausnitzii is one of the most abundant bacteria in a healthy human gut (Moore and Holdeman, 1974; Wang et al., 1996), and has recently been suggested to have a positive effect on the human gut health (Sokol et al., 2009). *F. prausnitzii* belongs to the *Clostridium leptum* group (*Clostridium* cluster IV), belonging to phylum *Firmicutes* (Lineage: *Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Faecalibacterium; Faecalibacterium prausnitzii*). *F. prausnitzii* has been previously called *Fusobacterium prausnitzii* (also cited as *F. prausnitzii* to avoid confusion), but physiological and phenotypic studies together with phylogenetic analyses based on the small subunit ribosomal ribonucleic acid (16S rRNA) gene sequencing have revealed that *F. prausnitzii* is only distantly related to *Fusobacteria* and is more closely related to members of *Clostridium* cluster IV (Duncan et al., 2002). The phylogenetic relations are still uncertain and constantly specified; for example, in a recent study by Lopez-Siles and co-workers (2012), *F. prausnitzii* species were divided into two separate phylogroups based on the 16S rRNA gene sequences.

1.2.1. Main features of *F. prausnitzii*

F. prausnitzii was first isolated in 1922 by C. Prausnitz. Morphologically, *F. prausnitzii* is a Gram-negative, non-motile and non-sporeforming rod with a diameter of 0.5 to 0.9 × 2.4 to 14.0 μm (Cato et al., 1974). *F. prausnitzii* is a strictly anaerobic bacterium that produces butyrate, formate, D-lactate and CO_2 but no hydrogen as fermentation products (Moore and Holdeman, 1974; Duncan et al., 2002). The G + C content of its DNA is found to range from 47 to 57 mol % (Duncan et al., 2002). *F. prausnitzii* growth is inhibited by acidic pH and bile salts (Lopez-Siles et al., 2012).

The amount of *F. prausnitzii* in the healthy human gut may be linked to diet. Mueller and co-workers (2006) have studied the age-related, gender-related and country-related differences in fecal microbiota; in their study, the level of *F. prausnitzii* was highest in the Swedish study group compared to Italian, German and French groups, and it was hypothesized this could be due to dietary habits. Inulin-derived prebiotics have been shown to significantly increase *F. prausnitzii* concentration in the gut, which also suspects the role of diet in the *F. prausnitzii* abundance (Ramirez-Farias et al., 2009).

1.2.2. Possible health impacts of *F. prausnitzii*

In the research by Li & co-workers (2008), *F. prausnitzii* was statistically linked to eight urinary metabolites: dimethylamine, taurine, lactate, glycine, 2-hydroxyisobutyrate, glycolate, 3,5-hydroxylbenzoate and 3-aminoisobutyrate. This suggests that *F. prausnitzii* may be functionally a highly active member of the gut microbiota. Possible health effects of *F. prausnitzii* are still somewhat uncertain and under constant research.

1.2.2.1. Anti-inflammatory effects

Studies have demonstrated that *F. prausnitzii* may have pronounced anti-inflammatory effects. Sokol and co-workers (2008) have studied the effect of *F. prausnitzii* in peripheral blood mononuclear cells and the results suggest that *F. prausnitzii* may induce an increased secretion of an anti-inflammatory cytokine interleukin 10, and a decreased secretion of pro-inflammatory cytokines like interleukin 12 *in vitro*.

Sokol and co-workers (2008) have also studied the effect of *F. prausnitzii* in the prevention of 2,4,6-trinitrobenzenesulphonic acid –induced colitis in mice. Both *F. prausnitzii* and its supernatant were found to induce an increased interleukin 10 and a decreased interleukin 12 and tumor necrosis factor –α production *in vivo*, and the daily administration of either *F. prausnitzii* or its supernatant led to marked attenuation of colitis. These findings suggest that *F. prausnitzii* may have a capability to suppress inflammation, and it is hypothesized that this is due to metabolite(s) secreted by *F. prausnitzii*. The identification of the active molecule(s) involved in this anti-inflammatory effect is under research (Sokol et al., 2008).

1.2.2.2. Inflammatory bowel disease (IBD)

Sokol and co-workers (2009) have compared the gut microbiota of healthy subjects and IBD patients, and discovered that phylum *Firmicutes* is more represented in the gut of healthy subjects compared to IBD patients, and that the number of *F. prausnitzii* is significantly higher in the gut of healthy subjects. It has also been proven by Manichanh and co-workers (2006) that the number of *Firmicutes* is significantly reduced in the gut microbiota of patients having CD. The results of these studies imply that the phylum *Firmicutes* and especially *F. prausnitzii* may be crucial to the gut homeostasis and disease protection.

In a recent study of the microbiota of CD patients that had undergone a surgery, the concentration of *F. prausnitzii* was much higher in the intestine of the patients that were still in remission six months after the surgery than in the intestine of the patients that had relapsed (Sokol et al., 2008). This suggests that a significant reduction in *F. prausnitzii* might potentially lead to an increased inflammation and disease activity in CD. Yet partly contradictory results have been gained also; in a research by Jia and co-workers (2010), the number of *F. prausnitzii* in the intestine of CD patients was found to be significantly lower than in the intestine of the healthy subjects, but surprisingly the successful treatment of CD seemed to result in a decrease instead of increase in *F. prausnitzii* abundance.

1.2.2.3. Butyrate production

Dietary fibers are fermented in the human colon by commensal bacteria to short-chain fatty acids (Nienstedt et al., 2004). Butyrate is the major product of carbohydrate fermentation in the colon. Butyrate modulates several processes and is a known anti-proliferative agent. In cultured cell lines, butyrate inhibits DNA synthesis and cell growth, mainly by inhibiting histone deacetylase (Kruh, 1982). Butyrate is also suggested to regulate the citric acid cycle, fatty acid oxidation, electron transport and TNF-α signaling (Vanhoutvin et al., 2009).

Animal studies have indicated that butyric acid may have antineoplastic properties, which means that it may protect against colon cancer (Bingham, 1990; Bradburn et al., 1993). It has therefore been hypothesized that dietary fiber is protective against colon cancer, because carbohydrates entering the large bowel stimulate the production of butyrate. Butyrate has also been suggested to provide protection against ulcerative colitis (Chapman et al., 1994).

F. prausnitzii is an important producer of butyrate, and the decrease of *F. prausnitzii* has been correlated to lower concentrations of fecal butyrate in healthy human subjects (Benus et al., 2010). Due to the protective nature of butyrate, it has been hypothesized that *F. prausnitzii* may have an important role in the protection of the colon (Chapman et al., 1994; Benus et al., 2010). Yet also contradictory results concerning the nature of butyrate

have been gained, and the benefits of butyrate are hence thought to depend on several aspects, such as time of exposure and butyrate amount (for review, see Lupton, 2004).

Benus and co-workers (2010) have investigated the association between *F. prausnitzii* and dietary fiber in healthy human subjects. Surprisingly, there was a statistically significant reduction in the *F. prausnitzii* abundance during both fiber-free and fiber-supplemented diets. Though, the reduction during fiber-supplemented diet could be due to the type of the supplement used in the study – the used supplement was pea fiber that may not support the growth of *F. prausnitzii* even though other fibers may do so. The study should thus be repeated with another fiber-supplement.

1.2.2.4. Energy intake

In both mice and humans, the relative abundance of *Bacteroidetes* and *Firmicutes* has been linked to obesity; the *Firmicutes* ratio is significantly higher in obese individuals (Ley et al., 2005; Bäckhed et al., 2004). It has also been shown that the transfer of the gut microbiota from an obese mouse to a germ-free mouse leads to significant increase in the fat mass of the recipient. A recent study by Balamurugan and co-workers (2010) proposes that high levels of *F. prausnitzii* might be linked to obesity in children. They examined the composition of fecal microbiota in obese and non-obese Indian children and found out that the levels of *F. prausnitzii* were significantly higher in obese children than in non-obese kids. These findings are contradictory to the observations by Liping Zhao, who used himself as a laboratory animal and monitored his gut microbes during his new diet based on Chinese fermented prebiotic foods (Hvistendahl, 2012). The *F. prausnitzii* level of Zhao's gut increased from undetectable level to 14.5 % of his total gut bacteria while he lost about 20 kilograms of weight.

It is conceivable that the high number of *F. prausnitzii* leads to higher energy intake, because *F. prausnitzii* is responsible for a significant proportion of fermentation of unabsorbed carbohydrates in the gut (Balamurugan et al., 2010), but understanding the possible link between the *F. prausnitzii* abundance and obesity needs more research.

1.3. Methods to examine the gut microbiota

The GI microbiota is an important element of the human body and is receiving constantly increasing attention. Especially the bacterial composition of the human gut has been under intensive research during recent years, driven by the huge Human Microbiome Project (The Human Microbiome Project Consortium, 2012) and MetaHIT project (Qin et al., 2010).

The traditional methods for identifying bacteria include bacterial cultivation, morphological examination and various biochemical tests. These methods are not quite suitable for studying gut microbiota, because a sizeable portion of gut bacteria are strict anaerobes and thus not cultivable (for review see Rajilic-Stojanovic et al., 2007). In addition, the traditional methods are relatively time-consuming and laborious. For these reasons, even though the traditional methods are cheaper, molecular biology methods have become a general method for detecting gut microbes

Molecular biology offers several rapid and specific methods for detecting and quantifying bacterial species, and these methods have revolutionized the understanding of the composition and metabolic activities of the gut microbiota (for review see Sartor, 2008). The study of genetic material extracted directly from environmental samples, referred to as metagenomics, enables studying the structure and also functions of microbial communities that cannot be cultivated (for review see Lepage et al., 2013). Metagenomics is thus an emerging field in studying the gut microbiota and the interactions between the microbes and the human host.

Even though molecular biology methods have revolutionized the gut microbiota research, the immense variation in the microbial composition between individuals makes it very difficult to define the ideal state of the gut (for review see Manichanh et al., 2012). If the "healthy composition" of the gut microbiota could be determined, it would be much easier to find the features that, when disrupted, associate with disease. For this reason, germ-free and gnotobiotic mice have been widely used when studying gut microbiota. When the presence and composition of the mice gut microbes is known and manipulated, the significance or effects of specific microbes can be studied. Mouse experiments have led to

great discoveries in, for example, autoimmune disease research (for review see Honda and Littman, 2012).

1.3.1. Polymerase Chain Reaction (PCR) based methods

One of the most widely used methods in studying gut microbiota is the traditional PCR amplification and sequence analysis of the 16S rRNA encoding genes (for review, see Lepage et al., 2013). Conventional PCR is fast, easy to perform and a relatively cheap method that is useful when detecting certain bacterium species or studying bacterial compositions. It does not, however, provide quantitative detection let alone give information about bacterial functions. While conventional PCR does not provide quantitative detection, real-time PCR with species-specific primers targeting 16S rRNA encoding genes can offer precise quantification of the target bacteria (Matsuki et al., 2004). In quantitative PCR (qPCR), the amount of PCR product in each PCR cycle is determined through measurement of fluorescence, and the quantity of the target bacteria can thus be calculated.

1.3.1.1. Genetic fingerprinting

Genetic fingerprinting techniques are widely used in comparing the microbial communities and in following the behavior of one specific community over time (for review see Muyzer, 1999). A general fingerprinting procedure includes DNA extraction from a sample, amplification of the genes encoding the 16S rRNA and analysis of the PCR products by denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) or corresponding technique. DGGE has been used in several gut bacteria studies, because it has advantages over other molecular approaches in that it is relatively inexpensive and has a high throughput (Noor et al., 2010). DGGE also enables the detailed characterization of the predominant bacteria by providing the possibility of sequencing the individual bands from the DGGE gel without cloning (for review see Muyzer and Smalla, 1997).

1.3.1.2. A defect in PCR methods

All the PCR based approaches in studying gut microbiota start with a DNA extraction step. Some recent microbiota studies have had partly contradictory results, and a recent study by

Maukonen and co-workers (2012) states that the different DNA extraction methods used in the studies may be the reason for the contradictions. The chosen DNA extraction method impacts strongly the relative amounts of different bacteria found from fecal samples, and for this reason, the results gained with different laboratories are not necessarily reliably comparable (Maukonen et al., 2012; Salonen et al., 2010).

1.3.2. Fluorescence *in situ* Hybridization (FISH)

In FISH, the studied bacterial cells are hybridized with species-specific fluorescent probes targeting 16S rRNA gene sequences, and the bacterial cells are then inspected with either fluorescent microscopy or flow cytometry (for review see Moter and Göbel, 2000). FISH avoids the possible selective effect of DNA extraction (1.3.1.2.), because in FISH, whole bacterial cells are analyzed (Vaahtovuo et al., 2005).

1.3.3. Feces as a sample material in gut microbiota studies

Fecal samples are often used to investigate human gut microbiota because they are relatively easy to collect; stool sample analysis offers a non-invasive way to evaluate the gut microbiota. Fecal samples are found to be quite good qualitative indicators of the distal colonic microbiota, but fecal microbes do not reflect that well to the mucosal gut microbiota as a whole (Holzapfel et al., 1998; Eckburg et al., 2005; Lyra et al., 2012). It is thus uncertain, how well the fecal bacterial composition reflects to the actual condition of the gut. Even though significant differences between fecal and mucosal communities have been discovered, it has been stated that feces would be a relevant sample type for at least studies concerning gut inflammation (Sokol et al., 2009).

2. AIM OF THE STUDY

The general purpose of this study was to examine the diagnostics and clinical significance of *Faecalibacterium prausnitzii* (*F. prausnitzii*), a commensal bacterium belonging to the human gut microbiota. One aim of this study was to set up a practical quantitative method for examining *F. prausnitzii* from fecal samples, to be used in the UTULab clinical laboratory and in the future studies focusing on the possible health marker status of *F. prausnitzii*. The other aim of this study was to cultivate *F. prausnitzii* and examine its antibiotic resistance. This study was part of a broader study concentrating on the possible health markers of the gut, and the main purpose was to make it possible to conveniently investigate *F. prausnitzii* from fecal samples in the future.

Specific aims of the study:

1. To validate an optimal DNA extraction method to be used in UTULab for extracting *F. prausnitzii* DNA from fecal samples.
2. To set up a practical quantitative method for examining *F. prausnitzii* from patient samples in UTULab.
3. To cultivate *F. prausnitzii* and investigate its resistance to some of the most common antibiotics.

3. MATERIALS AND METHODS

All the laboratory work in this study was performed following the general safety and laboratory practices of UTULab. DNA extraction, traditional PCR, qPCR and FISH were performed at the Department of Medical Microbiology and Immunology, University of Turku. The cultivation on *F. prausnitzii* was done in the premises of the Institute of Dentistry, University of Turku.

For this study, fresh stool samples were obtained from healthy volunteers with no notable GI symptoms. All volunteers gave permission for using their stool samples in research purposes. In addition, frozen stool samples were gained from the MOTOSD study conducted at the University of Jyväskylä, Department of Health Sciences in 2012. Ethical approval for using these samples in research purposes had been granted in advance by the Ethics Committee of the Central Finland Health Care District (7U/2011, 26.4.2011), and an informed consent was obtained from all subjects prior to the assessments.

3.1. DNA extraction

Two different fecal DNA extraction methods were compared to find out the most practical to be used in the UTULab laboratory. The compared methods in this study included a manual method (QIAamp DNA Stool Mini Kit, Qiagen, UK) and a semiautomatic method (GXT Stool Extraction Kit VER 2.0, Hain Lifescience GmbH, Germany). QIAamp DNA Stool Mini Kit is a commercial DNA extraction kit that is based on cell lysis in a lysis buffer, absorption of DNA inhibitors with InhibitEX matrix and DNA purification on spin columns (QIAamp DNA Stool Handbook. 2nd Edition. 04/2010). The method has been widely used in recent gut microbiota studies. GXT Stool Extraction Kit is an extraction kit intended to be used together with GenoXtract instrument (Hain Lifescience GmbH, Germany) for stool samples and rectal smears. The method is based on magnetic bead technology (GXT Stool Extraction Kit Ver 2.0 Instructions for Use. 12/2011).

First, the basic DNA extraction methods were compared with fresh and frozen stool samples. Then the methods were combined with an extra homogenization with MO BIO PowerLyzer™ 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, Inc.,

USA) to see if there was any difference in the DNA gain due to the intensive mechanical lysis. All the extracted DNAs were stored at -20˚C. In text and tables, QIAamp DNA Stool Mini Kit is referred to as QIA and GXT Stool Extraction Kit as GXT. The extra homogenization with MO BIO PowerLyzer™ 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, Inc., USA) is referred to as bead-beating (BB).

3.1.1. QIA

Bacterial DNA from 200 mg aliquot of fecal sample was extracted using QIAamp DNA Stool Mini Kit (Qiagen, UK) according to the manufacturer's instructions (QIAamp DNA Stool Handbook. 2nd Edition. 04/2010). The first incubation step on a heat block (Techne Dri-Block® DB-3D) was done at 95˚C instead of 70˚C to make the cell lysis more efficient.

When performing QIA combined with bead-beating, 200 mg aliquot of fecal sample was added to an eppendorf tube with 1.4 ml of ASL buffer (Qiagen, West Sussex, UK) and homogenized with PowerLyzer™ 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, Inc., USA) at 2000 rpm for 5 minutes. After that, the DNA extraction was done similarly as the basic QIA method.

3.1.2. GXT

DNA from 100 mg aliquot of fecal sample was extracted with GXT Stool Extraction Kit VER 2.0 (Hain Lifescience GmbH, Germany). The DNA extraction was, with the exception of the sample mass, done by following the manufacturer's instructions (GXT Stool Extraction Kit Ver 2.0 Instructions for Use. 12/2011).

When combining GXT with bead-beating, the DNA extraction was otherwise done as the basic GXT, but the first 5 minute vortexing of the manufacturer's instructions was replaced with a homogenization with PowerLyzer™ 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, Inc., USA) at 2000 rpm for 5 minutes.

3.1.3. DNA extraction from control bacterial strains

Total DNA was extracted from several control bacterial strains (Table 1) with a simple Chelex® method that is routinely used in UTULab for extracting DNA from bacterial cells growing on agar plates (UTULab Quality Manual 2013). The control samples were for traditional PCR and qPCR, in which *F. prausnitzii* DSM 17677 was used as a positive control and the others were used as negative controls.

Table 1: Control strains used for PCR and qPCR

Bacterial strain	ATCC number	Cultivation atmosphere	Agar plate
Faecalibacterium prausnitzii	DSM 17677	Anaerobic, +37˚C	FAA-blood
Bacteroides fragilis	Old patient sample	Anaerobic, +35˚C	FAA-blood
Bifidobacterium adolescentis	ATCC 15703	Anaerobic, +35˚C	FAA-blood
Lactobacillus acidophilus	ATCC 4356	Anaerobic, +35˚C	FAA-blood
Streptococcus salivarius	ATCC 19258	Aerobic, +35˚C	Blood
Enterococcus faecalis	ATCC 29212	Aerobic, +35˚C	Blood
Staphylococcus epidermidis	ATCC 14990	Aerobic, +35˚C	Blood
Escherichia coli	ATCC 25922	Aerobic, +35˚C	Blood
Pseudomonas aeruginosa	ATCC 27853	Aerobic, +35˚C	Blood
Corynebacterium diphtheria	ATCC 11050	Aerobic, +35˚C	Blood
Streptococcus pyogenes	ATCC 8184	Aerobic, +35˚C	Blood
Salmonella typhimurium	ATCC 13311	Aerobic, +35˚C	Blood

The selected bacterial strains were grown overnight on either blood agar or Fastidious Anaerobe Agar (FAA)-blood plates made by UTULab (UTULab Quality Manual 2013). An exception was *F. prausnitzii* that was cultivated for three days. After visual verification that a pure culture of a bacterium strain had grown, 1 µl of the cultivated cells was put into an eppendorf tube with 1 ml of 5 % Chelex® (Appendix I). Samples were vortexed and then incubated on a 100˚C heat block for 30 minutes. After the incubation, hot samples were vortexed briefly and then centrifuged for 12 minutes at 11 000 rpm in a table-top centrifuge 5414.D (Eppendorf Ag., Germany). 600 µl of supernatant was transferred to a new eppendorf tube and stored.

3.1.4. Measuring the DNA purity and concentration

The purity and concentration of the extracted DNA samples were measured using a NanoDrop ND-1000 spectrophotometer (ND, Nanodrop Technologies Inc., USA) at 260 nm. Kits' elution buffers were used as blanks for QIA and GXT samples and 5 % Chelex® supernatant for control samples.

3.1.5. Reproducibility study

The repeatability of the DNA extraction methods was compared by performing three parallel DNA extractions from two fecal slurries made from fresh stool samples (Figure 3). The slurries were made by weighing the sample, adding H_2O in ratio 1:1, vortexing and then homogenizing the sample thoroughly with Ultra-Turrax® T 25 dispersing instrument (Janke & Kunkel GmbH & Co. KG, IKA-Works) for 30 seconds.
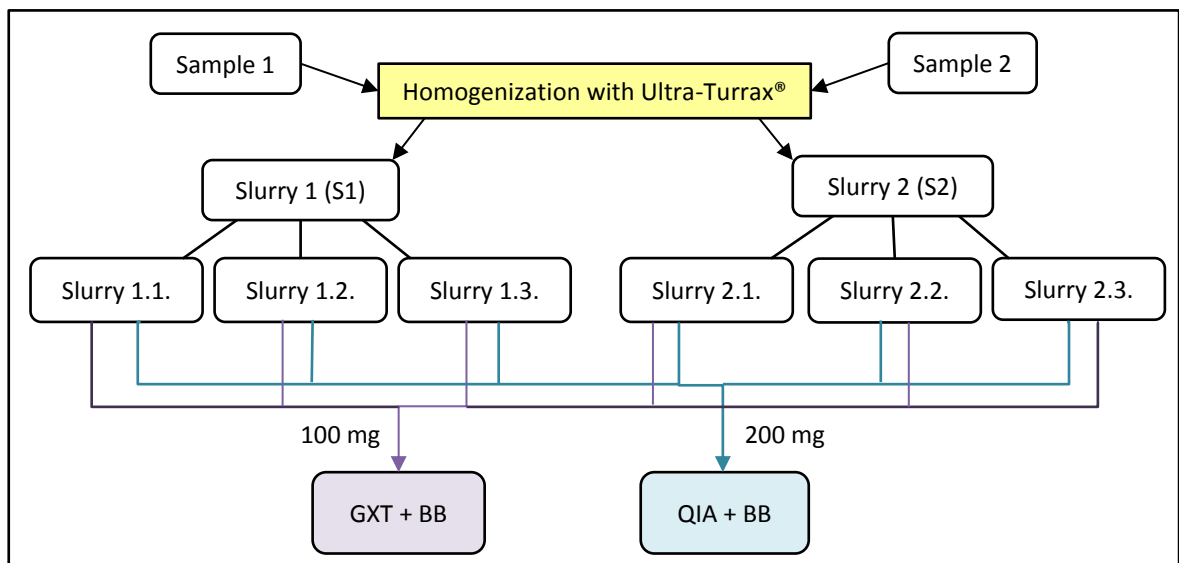
Figure 3: Reproducibility study. GXT = GXT Stool Extraction Kit, QIA = QIAamp DNA Stool Mini Kit, BB = bead-beating.

The reproducibility study was repeated after storing the homogenized samples at -70°C for one week, to see the possible effect of freezing on DNA quantity and quality.

3.2. PCR amplification of *F. prausnitzii*

After the comparison of the DNA extraction methods, the most suitable protocol was used for extracting DNA from the final study cohort (Table 2). The cohort consisted of fecal samples from adult volunteers. Most of these samples had been collected at the University of Jyväskylä, Department of Health Sciences. Some of the extracted DNAs were first used in validating the PCR methods, and then the whole sample set was analyzed with qPCR.

Table 2: Fecal samples used in this study (*n* = 27)

Sample code	Age	Gender	Sample collection	DNA extraction and cell fixation
FP4c	35	F	1.11.2012	1.11.2012
FP5	33	M	8.11.2012	8.11.2012
FP16a	58	F	16.10.2012	13.11.2012
FP16b	58	F	18.10.2012	13.11.2012
FP17a	65	M	17.10.2012	13.11.2012
FP17b	65	M	11/2012	13.11.2012
FP18a	33	M	4.11.2012	13.11.2012
FP18b	33	M	12.11.2012	13.11.2012
FP20	45	M	n/a	22.11.2012
FP21	53	M	n/a	22.11.2012
FP22	41	M	n/a	22.11.2012
FP23	34	M	n/a	22.11.2012
FP24	54	M	n/a	22.11.2012
FP25	35	M	n/a	22.11.2012
FP26	40	M	n/a	22.11.2012
FP27	53	M	n/a	22.11.2012
FP28	29	M	n/a	22.11.2012
FP29	53	M	n/a	22.11.2012
FP32	59	M	4.11.2012	18.12.2012
FP34	35	M	n/a	18.12.2012
FP35	65	M	23.11.2012	18.12.2012
FP36	55	M	4.12.2012	18.12.2012
FP37	57	M	10.12.2012	18.12.2012
FP38	56	M	4.5.2012	18.12.2012
FP39	61	M	6.3.2012	18.12.2012
FP40	51	M	n/a	18.12.2012
FP41	57	M	9.3.2012	18.12.2012

3.2.1. Traditional PCR

Traditional PCR was performed using *F. prausnitzii* 16S rRNA –targeted primers that had been previously designed and approved by Sokol and co-workers (2009; Table 3). Primers were ordered from Thermo Scientific (Thermo Fisher Scientific, Inc., Germany) and 10 μM stock solutions of the primers were made.

Table 3: *F. prausnitzii* primers used for both conventional PCR and qPCR.

Primer name	Sequence
Fprau 07	5'-CCA TGA ATT GCC TTC AAA ACT GTT-3'
Fprau 02	5'-GAG CCT CAG CGT CAG TTG GT-3'

For traditional PCR, AmpliTaq Gold™ DNA polymerase enzyme (Applied Biosystems, USA) and 10x PCR buffer containing 15 mM $MgCl_2$ (Applied Biosystems, USA) were used. As deoxyribonucleotide triphosphates, a 1 mM stock solution made from the 10 mM dNTP mix (Promega Biotech AB, Sweden) was used.

The reaction mixture for one PCR reaction is represented in Table 4. The mixture had a final concentration of 0.2 μM of each primer, 100 μM of dNTPs and 1.5 U of the polymerase enzyme. All the fecal DNA samples were diluted 1:10 before pipetting.

Table 4: Reaction mixture for one PCR reaction

Reagent	Volume (μl)
10x Buffer containing 15 mM $MgCl_2$	5.0
Fprau 07 primer (10 μM)	1.0
Fprau 02 primer (10 μM)	1.0
dNTP mix (1 mM)	5.0
AmpliTaq Gold™ polymerase enzyme	0.3
Aqua	36.7
Total	**49.0**
DNA template	1.0

The PCR program with ''Vertti'' (Veriti 96 Well Thermal Cycler, Applied Biosystems, USA) consisted of pre-incubation at 95ºC for 10 minutes, 40 cycles at 95ºC for 30 seconds, 60ºC for 1 min and 72ºC for 30 seconds and then a final extension at 72ºC for eight minutes. The expected PCR product size was 140 bp.

The PCR products were checked with Agarose Gel Electrophoresis (AGE) to confirm that a single, correct-size band was obtained. 1.5 % GellyPhor gels including 4 μl Midori Green Advance DNA stain (Biotop Oy, Finland) per 120 ml gel were used. 8 μl of PCR product

was mixed with 2 μl of Crystal 5x DNA loading buffer blue (Bioline GmbH, Germany), and from this suspension 6 μl was pipetted into the gel wells. Either Invitrogen 100 bp DNA ladders (0.25 μg/μl) or HyperLadder™ 100 bp ladders (Bioline GmbH, Germany) were used as standards.

The AGE run in 1x TBE buffer took about 2 hours at 40 mA / ~65 V in AGE chamber HU15 Midi Horizontal (Scie-Plas Ltd., England) and with Consort EV222 electrophoresis power supply (Consort nv., Belgium). Gels were visualized under the UV light using UVP BioSpectrum® AC Imagine System device and Vision Works computer program. Gel pictures were printed.

3.2.2. Sequencing PCR

Some of the *F. prausnitzii* PCR and qPCR products were sequenced to confirm that the gained products were indeed *F. prausnitzii*. In addition, the identity of some of the cultivated *F. prausnitzii* colonies was confirmed by sequencing; Chelex® extracted DNA from the cultivated bacteria was used as a template for universal 16S rRNA PCR (performed by UTULab routine lab worker Kirsi Sundholm-Heino), and the universal PCR products were sequenced.

The sequencing reactions were prepared by UTULab routine lab workers Tarja Kyrölä and Tarja Laine. First, the PCR products were purified with Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, USA). The DNA concentrations were then measured with NanoDrop ND-1000 spectrophotometer (ND, Nanodrop Technologies Inc., USA) and the sequencing reactions (template + primer dissolved in 6 μl of H_2O) were prepared. The primer for *F. prausnitzii* PCR and qPCR products was Fprau 02 (Table 3) with the concentration of 3.2 pmol per reaction. The primer for universal PCR products was forwCLSI 5'-TTG GAG AGT TTG ATC MTG GCT C-3' (UTULab Quality Manual 2013) with the concentration of 3.2 pmol per reaction. The sequencing PCR was performed at the DNA Sequencing Service of The Finnish Microarray and Sequencing Centre, Turku Centre for Biotechnology. The sequences were analyzed with the Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, available at http://blast.ncbi.nlm.nih.gov).

3.2.3. Quantitative PCR (qPCR)

Quantitative PCR was performed with the same primers and in the same PCR conditions as the traditional PCR (3.2.1.). LightCycler® 480 II Real-Time PCR System (Roche Applied Science, Germany) was used for performing the reactions, and ready-to-use LightCycler® 480 SYBR Green I 2x Master Mix (Roche Diagnostics GmbH, Germany) was used. All the fecal DNA samples were diluted either 1:100 or 1:500 before qPCR. The reaction mixture for one qPCR reaction is presented in Table 5. For each sample well on a 96-well plate, 22 µl of the reaction mixture and 3 µl of diluted template were pipetted. Sample plate was quickly centrifuged with Mini Plate Spinner MPS 1000 (Labnet International, Inc., USA) before inserting the plate to the machine and starting the program.

Table 5: Reaction mixture for one qPCR reaction

Reagent	Volume (µl)
SYBR Green Master Mix (2x)	12.5
F. prausnitzii 02 primer (10 µM)	0.5
F. prausnitzii 07 primer (10 µM)	0.5
Aqua	8.5
Total	**22.0**
DNA template	3.0

The dilution series for the standard curve was made from the pool of positive reaction products of the previously performed traditional PCR. The DNA concentration of the DNA pool was measured with NanoDrop ND-1000 spectrophotometer (ND, Nanodrop Technologies Inc., USA) at 260 nm. H_2O was used as a blank. Assuming that the molecular weight of one DNA base pair is 660 g/mol and knowing the PCR product size to be 140 base pairs, the concentration of DNA fragments in the pool could be calculated based on the DNA concentration:

$$fragment\ size\ (bp) \times 660\ g/mol =\ molecular\ weight\ of\ one\ fragment\ (g/mol)$$

$$\frac{DNA\ concentration\ (ng/\mu l)}{molecular\ weight\ of\ one\ fragment\ (g/mol)} = fragment\ concentration\ (nmol/\mu l)$$

When the fragment concentration (mol/µl) was multiplied with Avogadro's number (6.0221415×10^{23}), the number of fragments per one µl was gained. From this, a dilution series from 10^{10} to 10^1 was done.

The qPCR results were analyzed with LightCycler® 480 Software (Roche Applied Science, Release 1.5.0 SP4). Melt curve analysis was used for result quality validation. The statistical analysis of the results was done with Microsoft Excel 2010 and IBM SPSS 21.0 software.

3.3. Fluorescence *in situ* Hybridization (FISH)

FISH combined with fluorescence microscopy analysis was performed to the same fecal samples that were analyzed with qPCR (Table 2). Several control bacterial strains were cultivated, fixed and used as negative controls. *Faecalibacterium prausnitzii* DSM 17677 was used as a positive control (Table 6).

Table 6: Control strains for FISH

Bacterial Strain	ATCC number	Cultivation atmosphere
Faecalibacterium prausnitzii	DSM 17677	Anaerobic, +37˚C
Bacteroides fragilis	Old patient sample	Anaerobic, +35˚C
Bifidobacterium adolescentis	ATCC 15703	Anaerobic, +35˚C
Lactobacillus acidophilus	ATCC 4356	Anaerobic, +35˚C
Salmonella typhimurium	ATCC 13311	Aerobic, +35˚C
Yersinia enterocolitica	Old patient sample	Aerobic, +35˚C

3.3.1. Collection of the control bacterial cells

Control bacterial strains (Table 6) were grown overnight either in 15 ml BD Falcon™ tubes with 5 ml Tryptic Soy Broth (TSB) or in Fastidious Anaerobe Broth (FAB) -tubes routinely manufactured by UTULab (UTULab Quality Manual 2013). An exception was *F. prausnitzii* that was cultivated for three days.

Cultivated suspensions were centrifuged at 4000 rpm for 10 minutes with IEC Centra® GP8R centrifuge (IEC International Equipment Company, USA). Supernatant was discarded and the cell pellet was suspended in approximately 10 ml of Phosphate-Buffered Saline (PBS). Centrifugation step (4000 rpm for 10 minutes) was repeated. Supernatant was discarded and the cell pellet was suspended in 1 ml of PBS. Suspensions were transferred to 2 ml eppendorf tubes and centrifuged at 13 200 rpm for 5 minutes in a table-top centrifuge 5414.D (Eppendorf Ag., Germany). Supernatant was removed and cells were stored at freezer -20ºC until fixation.

3.3.2. Fixation of the control cells

Control bacterial cells were fixed for FISH by suspending the cell pellet (see 3.3.1. for collection of the cells) in 400 µl of PBS and 1200 µl of 4 % paraformaldehyde (Appendix I). The suspensions were incubated overnight at +4ºC with gentle shaking. Next morning, the fixation suspensions were centrifuged at full speed (13 200 rpm) for 5 minutes. Supernatant was discarded to paraformaldehyde waste and the cell pellet was washed three times with PBS: the pellet was suspended to 1 ml of PBS, centrifuged at 13 200 rpm for 3 minutes and the supernatant was discharged. After repeating the PBS-wash for three times, the cells were mixed in 250 µl of PBS + 250 µl of 96 % ethanol. A few sterile glass beads were added to the eppendorf tubes, and the tubes were stored at -20ºC. The cells were now ready for FISH analysis.

3.3.3. The collection and fixation of bacterial cells from fecal samples

The collection and fixation of fecal bacterial cells for FISH analysis were done as previously described by Vaahtovuo and co-workers (2005). The fixed bacterial cells were moved to 2 ml eppendorf tubes and suspended in 500 µl of PBS + 500 µl of 96 % ethanol. Sterile glass beads were added to the eppendorf tubes that were then stored at -20ºC until analyzed.

3.3.4. Hybridization

Both the fixed control bacterial cells and fecal bacterial cells were hybridized for microscope analysis in liquid phase with a Cy3 labeled F. prausnitzii 645 probe with the sequence 5'-CCT CTG CAC TAC TCA AGA AAA AC-3' (Suau et al. 2001, Thermo Fisher Scientific, Inc., Germany). The concentration of the probe stock was 1 µg/µl, from which 100 ng/µl dilutions were prepared and stored at -20ºC. Probe solutions were always kept protected from light.

Hybridizations were performed in 1.5 ml eppendorf tubes. All the fecal bacterial cell suspensions were diluted either 1:20 or 1:40 in PBS before hybridization; dilution factor depended on the visually inspected density of the sample. Control samples were not diluted. The total volume of each hybridization reaction was 100 µl. First, 5 µl of fixed

bacterial cells was mixed with 92.5 µl of hybridization solution (Appendix I). Before pipetting, the hybridization solution had to be warmed up to + 37ºC to dissolve the flaky SDS. The bacterial cells were vortexed thoroughly to break up any aggregates before pipetting to the hybridization solution. After pipetting the cells, samples were vortexed and the pre-hybridization was done by incubating the samples for 1 h in a water bath (Julabo TWB 5) at + 50ºC.

After the pre-hybridization, samples were thoroughly vortexed and then 2.5 µl of the thawed 100 ng/µl F. prausnitzii645 probe solution was added. Samples were again vortexed and then incubated overnight in a water bath at + 50ºC (covered from light). The hybridization time was about 18 hours.

The hybridized cells were washed and stained with DAPI (4,6-diamidino-2-phenylindole, Molecular Probes, USA) by pipetting 15 µl of the cells into 4 ml of pre-warmed DAPI wash solution (Appendix I). The solutions were then incubated for 30 minutes at 50ºC (covered from light), after which the samples were first vortexed and then sonicated with a SONOPULS HD 2070 sonicator (Bandelin electronics, Germany) for 5 seconds at 17% power to remove any aggregates. The sonicated bacterial cells were filtered onto Isopore™ 0.2 µm GTBP polycarbonate filters (Millipore, Ireland), washed with 4 ml of pre-warmed PBS, put on objective glasses and mounted with 8 µl of glycerol-PBS 9:1. (Vaahtovuo et al. 2005.)

3.3.5. Microscopy analysis

Bacterial cells were observed with Olympus BX 51 fluorescence microscope (Olympus Optical, Ltd., Japan) at 1000x magnification. The excitation/emission maxima for Cy3 dye is 550/570 nm and for DAPI 358/461 nm, so two different Olympus filters (U-MWIG2 for Cy3 and U-MWU2 for DAPI) were used in turn to view the cells. U-25ND25 neutral density filter was used to delay the color fading.

About 50 bacterial cells were visible in each field of vision with U-MWU2 filter. Bacteria from 10 fields of vision were counted per sample, and average values of the bacterial counts with both filters were calculated with Microsoft Excel 2010. Based on these results,

the *F. prausnitzii* proportion and the total bacterial quantity and *F. prausnitzii* quantity per 1 g of feces (wet weight) were calculated. Digital images from some of the samples were taken with Olympus DP70 microscope camera (Olympus Optical Co. Ltd., Japan). The final results were analyzed and compared with qPCR results with Microsoft Excel 2010 and IBM SPSS 21.0 software.

3.4. Cultivation of *F. prausnitzii*

The cultivation of *F. prausnitzii* was performed from a reference strain DSM 17677 (Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany). The bacterial tube was opened in a Whitley A35 anaerobic workstation (Don Whitley Scientific, UK) at the Institute of Dentistry, University of Turku, and the bacterial suspension was transferred into various broths and agar plates (Table 7) manufactured by UTULab (UTULab Quality Manual 2013). The remaining of the bacterial suspension was taken out from the anaerobic workstation and transferred to the same broths and agar plates outside the chamber. These were put in an anaerobic jar to grow. Possible colonies were visually inspected after four days of cultivation.

After the successful cultivation, the bacterial cultures were sustained by transferring them to new broths and agars once or twice a week. Several agars and broths (Table 7) were tested to find the most optimal growth medium for *F. prausnitzii*. Also the freezing and thawing of the bacterial cells was tested several times with different conditions. Gram-staining, PCR and sequencing were performed regularly to confirm that no contaminations had occurred.

Table 7: Agars and broths used in the *F. prausnitzii* cultivation study

Agars and broths used for the initial cultivation of DSM 17677	Additional agars and broths tested after the successful initial cultivation
FAA-blood	FAA + Cb + Gluc
CYE agar	Wilkins-Chalgren agar
FAB	FAB + Cb
FAB + BHI	FAB + Gluc
FAB + meat extract	FAB + Cb + Gluc
BHI	BHI + Cb
BHI + meat extract	BHI + Gluc
Meat extract	BHI + Cb + Gluc

All the broths and agars were made by UTULab. CYE agar = legionella agar; Wilkings-Chalgren agar = Anaerobic agar (Oxoid ltd, England); BHI = Brain-Heart Infusion broth; Cb = 0.5 % w/v Cellobiose; Gluc = 0.5 % w/v Glucose.

4. RESULTS

4.1. DNA extraction

The total DNA from fecal samples was extracted with two parallel methods. The DNA quantities and qualities obtained with the two DNA extraction methods were measured with Nano-Drop ND1000 spectrophotometer (ND, Nanodrop Technologies Inc., USA) at 260 nm and are represented in Table 8. To ensure the best reliability, only samples for which the two compared extraction protocols have been performed simultaneously, are presented and compared. The absorbance ratio 260/280 for pure DNA is around 1.80. The 260/230 ratio for pure nucleic acid is expected to be higher than the respective 260/280 value, commonly 2.0 – 2.2. (T042 Technical Bulletin, Thermo Fisher Scientific – NanoDrop Products, USA.)

Table 8: DNA quantities and qualities after the compared DNA extraction methods, measured with Nano-Drop ND1000 spectrophotometer (ND, Nanodrop Technologies Inc., USA) at 260 nm.

Sample code	GXT			QIA		
	DNA conc. (ng/µl)	260/280	260/230	DNA conc. (ng/µl)	260/280	260/230
TEST1	27.60	1.83	0.76	8.70	1.79	1.38
TEST2	42.70	1.70	0.92	20.70	1.81	1.06
FP1 fresh	48.00	1.73	0.58	27.30	2.02	2.18
FP1 frozen	53.50	1.75	0.65	14.80	1.82	1.47
FP2	94.10	1.84	1.12	36.00	2.02	2.00
	GXT + BB			QIA + BB		
FP4c	236.8	1.83	1.42	47.00	1.87	1.93
FP16a	134.50	1.82	1.21	24.60	2.04	2.22
FP16b	286.10	1.81	1.55	30.30	1.86	2.68
FP17a	176.90	1.94	1.60	79.20	2.04	2.51
FP17b	304.10	1.89	1.47	53.80	1.96	1.60
FP18a	579.70	1.74	1.04	78.90	1.99	2.21
FP18b	332.60	1.72	1.08	47.30	1.95	1.82

The DNA concentrations of five samples after basic DNA extractions are presented in the upper part of the table, while the DNA concentrations of seven samples after extraction protocols combined with an extra homogenization are presented in the lower part of the table. GXT: GXT Stool Extraction Kit, QIA: QIAamp DNA Stool MiniKit, BB: bead-beating with MO BIO homogenizer.

A positive correlation exists between the total DNA concentrations gained with GXT and QIA ($R = 0.750$; $P = 0.003$), but as can be seen from Table 8, the gained DNA quantity (ng/µl) is significantly higher in samples extracted with GXT than in samples extracted with QIA ($P = 0.002$). The average ratio of the total DNA quantity (ng/mg feces) with QIA

compared to GXT is only 13.8 %, meaning that the average DNA gain with GXT is almost seven times higher than with QIA. The 260/230 ratios are notably lower in samples extracted with GXT, indicating that the DNA quality is better in samples extracted with QIA. Samples presented in the bottom half of Table 8 (FP4c, FP16a, FP16b, FP17a, FP17b, FP18a and FP18b) have been analyzed with qPCR; these results are shown in Figure 6.

4.1.1. Reproducibility study

The reproducibility study was performed to compare the repeatability of the two DNA extraction methods. The results of the study are represented in Table 9. The gained DNA concentrations are significantly higher in samples extracted with GXT ($P = 0.015$). In addition, the 260/230 ratios of the samples extracted with GXT are a bit higher, but no statistical differences in the DNA qualities were observed. Only minor variations between the three parallel extractions have occurred when studying fresh stool samples (C.V. columns in Table 9), but the variation is increased in frozen samples. This can be merely a coincidence though, since only two sample sets were analyzed.

Table 9: Variation in DNA quantity and quality between three parallel DNA extractions.

Sample code	DNA concentration (ng/µl) with GXT				DNA concentration (ng/µl) with QIA			
	Average	S.D.	Var.	C.V. (%)	Average	S.D.	Var.	C.V. (%)
S1 fresh	100.52	7.02	49.31	6.99	11.53	1.13	1.27	9.77
S1 frozen	104.93	16.38	268.30	15.61	21.10	2.55	6.49	12.07
S2 fresh	60.30	3.34	11.16	5.54	10.63	0.37	0.14	3.46
S2 frozen	53.07	5.52	30.43	10.39	16.90	7.92	62.69	46.85
	260/280 GXT				260/280 QIA			
Sample code	Average	S.D.	Var.	C.V. (%)	Average	S.D.	Var.	C.V. (%)
S1 fresh	1.97	0.01	0.00	0.59	1.96	0.04	0.00	2.29
S1 frozen	2.00	0.02	0.00	1.03	2.04	0.04	0.00	1.97
S2 fresh	1.99	0.01	0.00	0.59	2.21	0.85	0.72	38.38
S2 frozen	1.99	0.00	0.00	0.00	2.05	0.04	0.00	1.96
	260/230 GXT				260/230 QIA			
Sample code	Average	S.D.	Var.	C.V. (%)	Average	S.D.	Var.	C.V. (%)
S1 fresh	1.79	0.04	0.00	2.16	1.45	0.31	0.10	21.39
S1 frozen	1.81	0.05	0.00	2.71	1.82	0.23	0.05	12.75
S2 fresh	1.61	0.03	0.00	1.68	1.44	0.19	0.04	13.33
S2 frozen	1.52	0.08	0.01	4.97	1.31	0.23	0.05	17.68

S.D. = standard deviation; Var. = variance; C.V. = coefficient of variation.

4.2. Traditional *F. prausnitzii* PCR

All the fecal DNA samples displayed a band on the AGE gel after PCR amplification with *F. prausnitzii* specific primers Fprau 07 and Fprau 02 (Table 3). The product of all the samples was of the expected size ~140 bp, indicating that all the studied fecal samples included *F. prausnitzii* 16S rRNA encoding DNA. The intensity of the bands varied, indicating that either the *F. prausnitzii* abundance or the PCR efficiency between the samples varied. Negative controls (Table 1) did not show bands. Figure 4 is representative of AGE results after the traditional *F. prausnitzii* PCR.
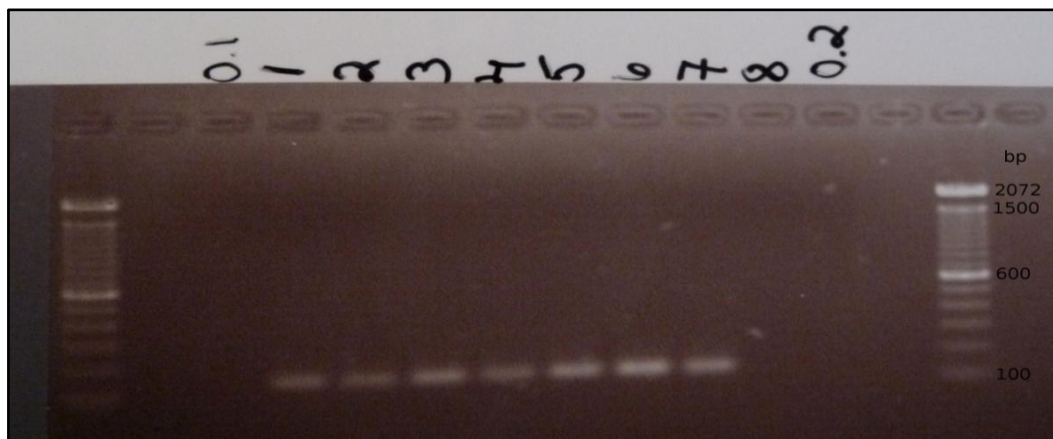

Figure 4: AGE gel picture after *F. prausnitzii* PCR performed on 8.1.2013. 1 = positive control (DSM 17677); 2 = Slurry 1.1 (1:1); 3 = Slurry 1.1 (1:10); 4 = Slurry 1.2 (1:1); 5 = Slurry 1.2 (1:10); 6: Slurry 1.3 (1:1); 7 = Slurry 1.3. (1:10); 8 = negative control including *Escherichia coli* DNA as template; 0.1 and 0.2 = blanks including aqua as template. Invitrogen 100 bp DNA ladders are used. All the fecal samples express a band of the expected size ~140 bp. Interestingly, the bands of the diluted samples 3 and 5 are a bit brighter than the bands of the corresponding undiluted samples 2 and 4.

After already purchasing the Fprau 07 and Fprau 02 primers, two mismatches were noticed in the Fprau 07 when comparing it to the 16S rRNA sequence of *F. prausnitzii* reference strain ATCC 27768 (one thymine and cytosine should have been in opposite positions). That did not seem to cause any trouble in the PCR, so new primers were not ordered.

4.3. Sequencing PCR

All the positive *F. prausnitzii* PCR products and all the cultivated *F. prausnitzii* colonies that were analyzed by sequencing at the DNA Sequencing Service of The Finnish Microarray and Sequencing Centre, Turku Centre for Biotechnology, were proven to be *F.*

prausnitzii. The two mismatches in the Fprau 07 primer were reproduced to the *F. prausnitzii* PCR products and lowered the alignment percentages, but it was still obvious that the gained PCR products and the cultivated bacteria were *F. prausnitzii*. This indicates that the PCR primers were working as expected and that no contaminations occurred during the cultivation study.

4.4. qPCR

Fecal samples FP4, FP16a, FP16b, FP17a, FP17b, FP18a and FP18b (see Table 8), together with fecal slurries S1.1, S1.2 and S1.3 ("S1 fresh" in Table 9), were analyzed with qPCR to see the effect of the extraction method on quantifying *F. prausnitzii* DNA from fecal samples. The results of the qPCR with *F. prausnitzii* primers Fprau 07 and Fprau 02 carried out on the different DNA extracts are shown in Figure 5. The results are represented as *F. prausnitzii* 16S rRNA gene copy numbers per one gram of feces (wet weight), and the represented results are average values of two parallel reaction wells. The average variation between the two parallel reactions was 3.4 %, indicating satisfactory reproducibility. It is noteworthy that the gene copy number does not directly represent the *F. prausnitzii* bacterial cell number of the sample, because it is not known how many gene copies of 16S rRNA exist in a single *F. prausnitzii* cell.
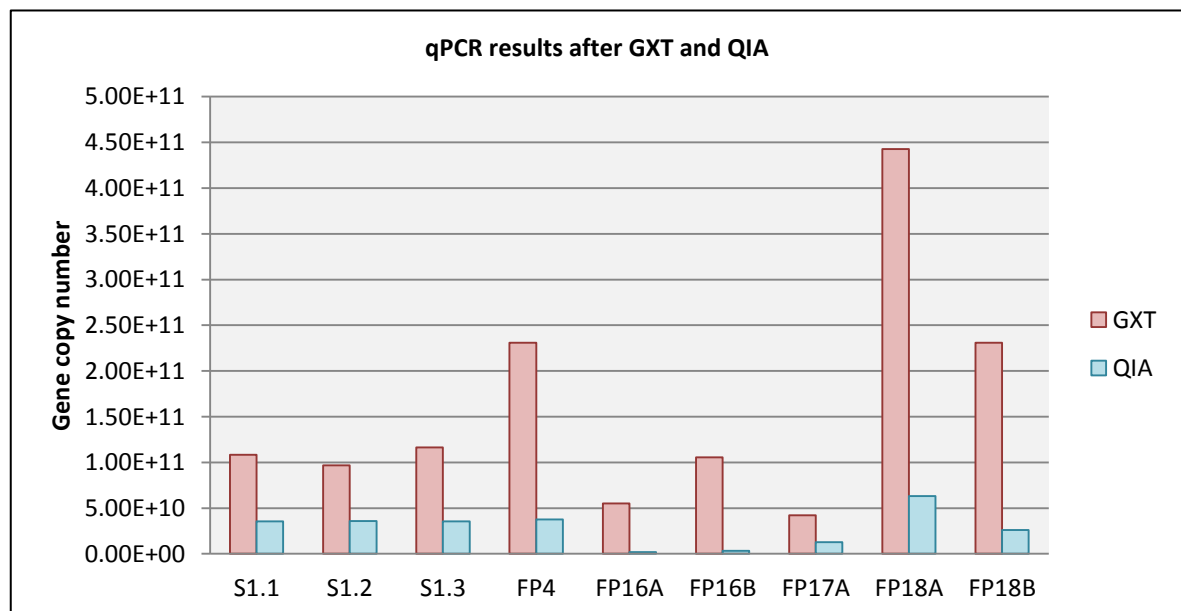

Figure 5: Differences in qPCR results after semiautomatic and manual DNA extraction methods. *F. prausnitzii* **16S rRNA gene copy numbers per 1 g of feces (wet weight) are shown. Two parallel reactions were analyzed per sample. FP17b was mistakenly excluded from the presented qPCR run and is thus not shown here.**

Positive correlation exists between the *F. prausnitzii* 16S rRNA gene copy numbers in the samples extracted with different methods ($R = 0.777$; $P = 0.005$), but the *F. prausnitzii* 16S rRNA gene copy numbers are significantly greater in the DNA samples extracted with GXT compared to the samples extracted with QIA ($P = 0.003$). Based on the comparison of the two extraction methods (Table 8, Table 9 and Figure 5), the DNA extraction for the rest of the final study cohort (Table 2) was decided to be performed with GXT method combined with bead-beating. The DNA extractions for samples FP4c – FP18b (Table 2) were not repeated, because GXT-extracted DNA of sufficient quality already existed for these samples. The DNA quantities and qualities of the whole final study cohort are presented in Appendix II, together with FISH and qPCR results.

The qPCR results for the final study cohort are presented in Figure 6. The results are represented as *F. prausnitzii* 16S rRNA gene copy numbers per one gram of feces (wet weight), and the represented results are average values of two parallel reaction wells. The *F. prausnitzii* 16S rRNA gene copy numbers (g^{-1} of feces) varied between 8.29×10^9 and 4.38×10^{11}, the difference between the extremes being over 50-fold. The analysis of possible interrelationships between the *F. prausnitzii* abundance and clinical characteristics of the study subjects was not included in this study.

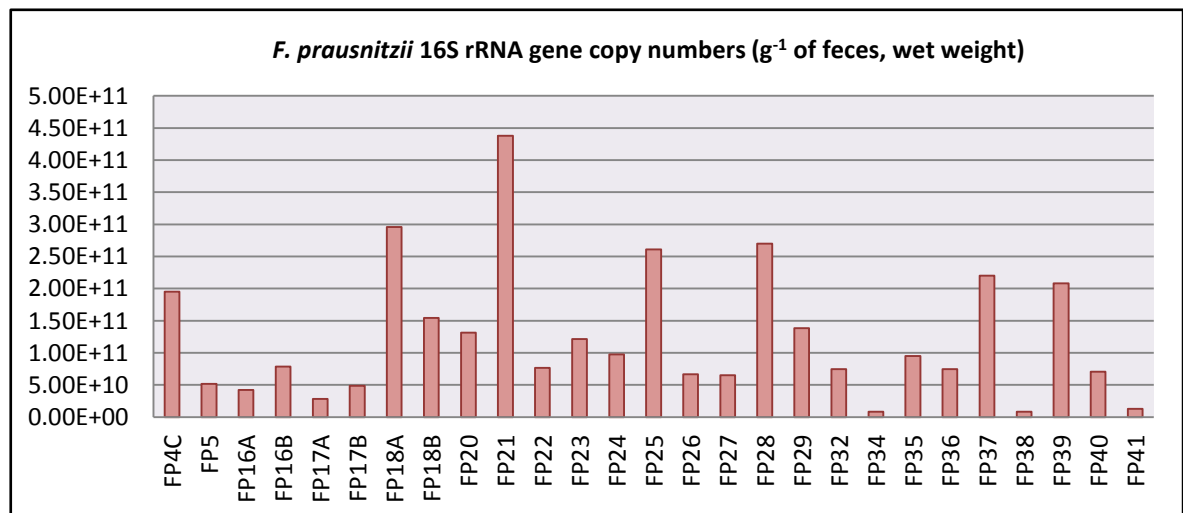**Figure 6: qPCR results for the final study cohort, represented as *F. prausnitzii* 16S rRNA gene copy numbers per one gram of feces (wet weight). The *F. prausnitzii* occurrence differs significantly between the studied fecal DNA samples.**

An example of qPCR melt curve analysis is represented in Figure 7. In this particular qPCR run, a dilution series of the positive control DSM 17677, a dilution series of the

standard DNA pool and a few fecal samples were analyzed. Two clearly separate melting peaks representing DSM 17677 and the DNA pool can be seen in the figure, indicating that the qPCR has led to two different products. The melting peaks of the fecal DNA samples are situated between the two main peaks, the situation depending on the sample.
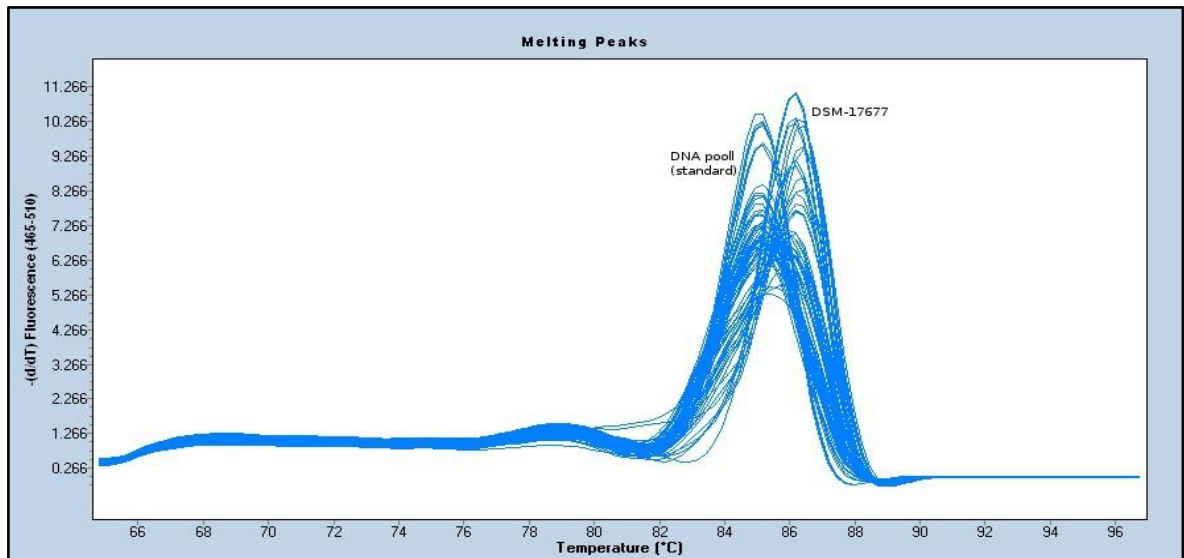

Figure 7: The melt curve analysis of a qPCR run performed on February 25th, 2013. The melting peaks of the standard DNA pool and DSM 17677 are clearly separated, meaning that the PCR products differ in their sequence. The melting peaks of the fecal DNA samples are situated in variable positions between the two main peaks.

The qPCR products of DSM 17677 and the DNA pool (Figure 7) were sequenced to find out the reason for the two separate melting peaks. Sequences of the qPCR products are aligned in Figure 8. The sequences are otherwise identical, but the sequence of the DNA pool includes an uncertain area "RY". This uncertainty in sequencing indicates that the DNA pool may include *F. prausnitzii* strains with differing DNA sequences.

Score	Expect	Identities	Gaps	Strand
167 bits(90)	3e-47	92/94(98%)	0/94(0%)	Plus/Plus

```
Query  1   CTCCCGATATCTACGCATTCCACCGCTACACCGGGAATTCCGCCTACCTCTGCACTACTC  60
           |||||||||||||||||||||||||||||||||||||||||  ||||||||||||||||||
Sbjct  4   CTCCCGATATCTACGCATTCCACCGCTACACCGGGAATTCCRYCTACCTCTGCACTACTC  63

Query  61  AAGAAAAACAGTTTTGAAGGCAATTCATGGAAAA  94
           ||||||||||||||||||||||||||||||||||
Sbjct  64  AAGAAAAACAGTTTTGAAGGCAATTCATGGAAAA  97


R = G/A
Y = T/C
```

Figure 8: Comparison of the sequences of DSM 17677 (Query) and the DNA pool used as qPCR standard (Sbjct). The two-base uncertain area of the pool sequence is highlighted.

4.5. FISH

Before analyzing the fecal samples with microscopy FISH, the optimal working of Cy3 stain compared with DAPI stain was confirmed with a positive control *F. prausnitzii* DSM 17677 (Figure 9). Ten fields of vision were counted, and the number of bacterial cells was found to be 99% identical with both dyes in all the fields of vision.
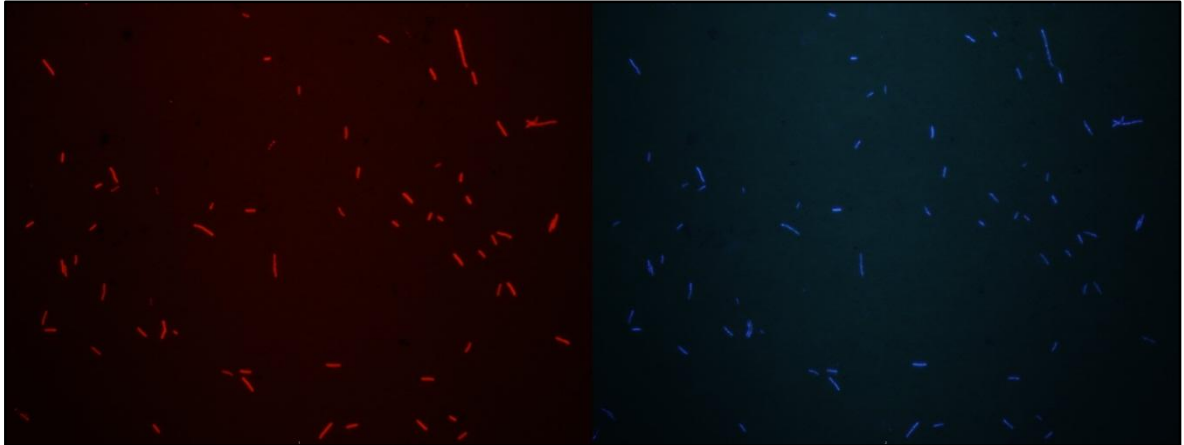

Figure 9: Montage of the positive control DSM 17677 stained with Cy3 (left) and DAPI (right). The number of *F. prausnitzii* cells was found to be equal with both stains. Image out of scale.

F. prausnitzii morphology in FISH was substantially different in fixed DSM 17677 samples compared to fixed fecal samples. The reference strain DSM 17677 (Figure 9) appeared as a relatively thick, medium-sized rod, while in fecal samples (Figure 10) the bacterium was a very short rod. One thing was noted characteristic for *F. prausnitzii* in almost every sample: the bacterium often appeared in pairs (Figure 11).
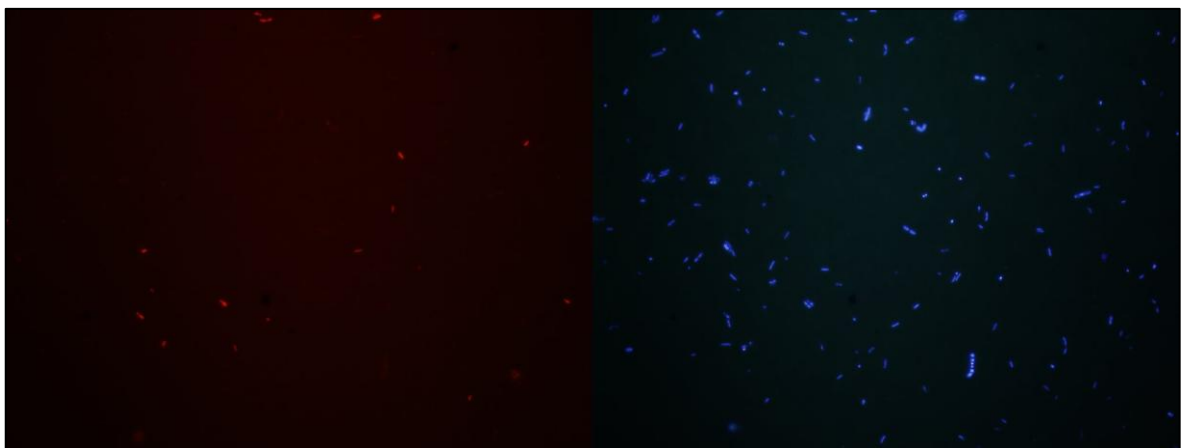
Figure 10: Montage of a fecal sample FP4c stained with Cy3 (left) and DAPI (right). As can be seen, the *F. prausnitzii* cells are substantially smaller than in the stained DSM 17677 sample (Figure 9). Image out of scale.
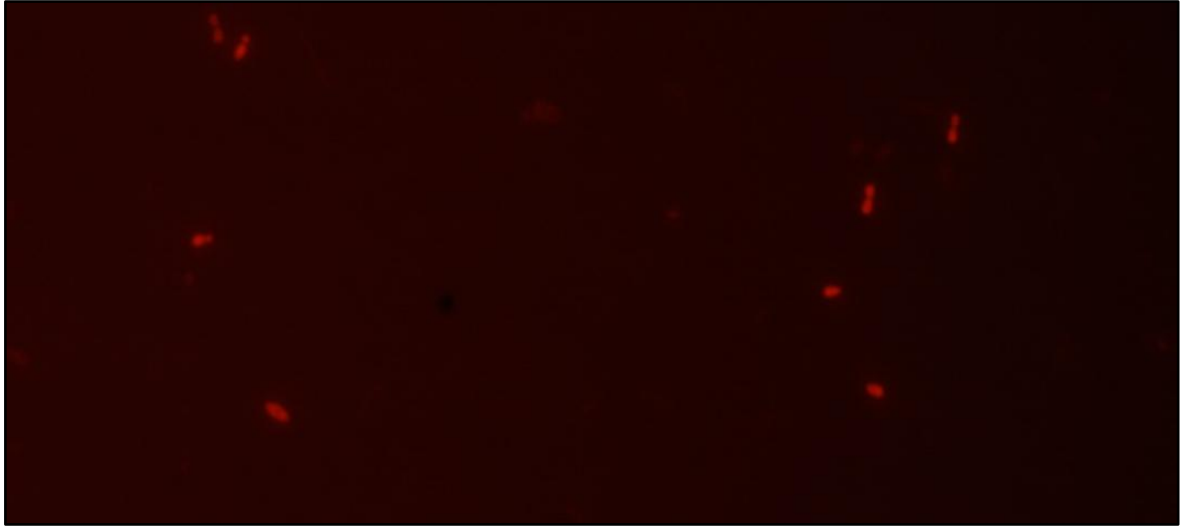
Figure 11: *F. prausnitzii* **stained with Cy3 (image out of scale). Presumably thirteen *F. prausnitzii* cells are visible in this image, most of them appearing in pairs.**

FISH results for the final study cohort are represented in Figure 11. Total bacterial cell number per one gram of feces (wet weight) and *F. prausnitzii* cell number per one gram of feces (wet weight) are shown. The presented results are average values calculated from ten fields of visions per sample.
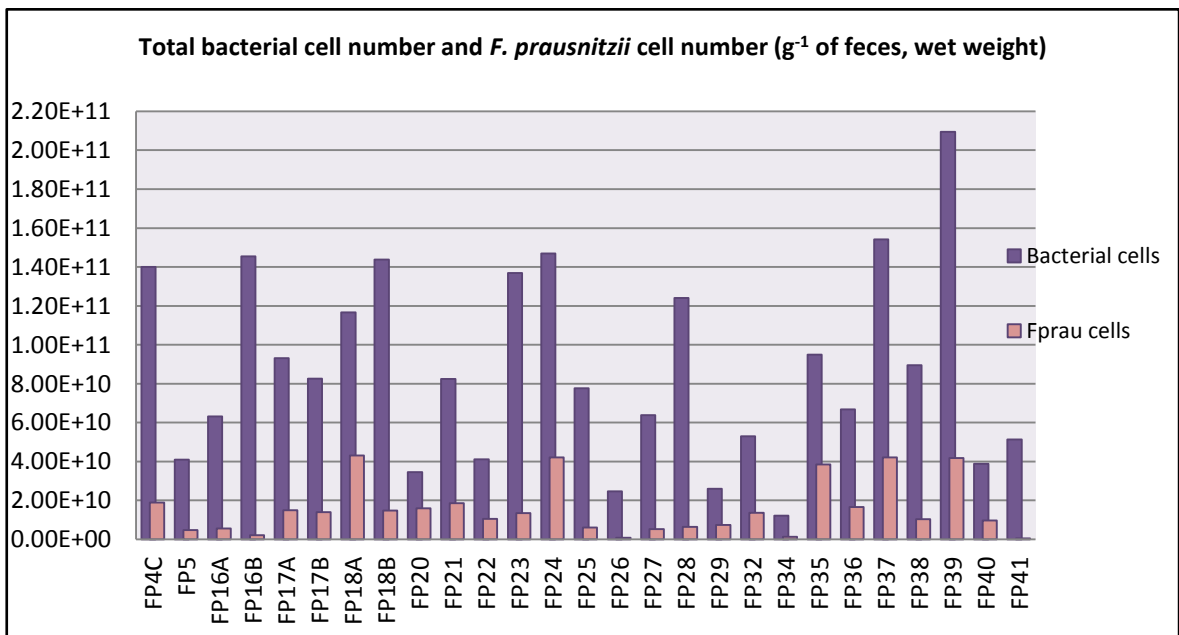
Figure 12: FISH results. Total bacterial cell number and *F. prausnitzii* cell number per one gram of feces (wet weight) are represented. Ten fields of visions were studied per sample; average values are presented.

Based on the FISH results, the total bacterial cell number of the studied samples (g^{-1} of feces) varies from 1.21×10^{10} to 2.09×10^{11}, and the *F. prausnitzii* cell number (g^{-1} of feces) varies between 5.51×10^{8} and 4.31×10^{10} (Figure 12; Appendix II). The relative *F. prausnitzii* abundances of the samples range from 1.08 % to 46.31 %, indicating large variations between individuals. The correlation coefficient between the total bacterial cell number and *F. prausnitzii* cell number in the studied fecal samples is 0.627 ($P \leq 0.001$), meaning that positive correlation exists between the bacterial cell counts and *F. prausnitzii* cell counts.

The DNA concentrations, FISH results and qPCR results of the final study cohort are summarized in Appendix II. The correlation coefficient between FISH and qPCR results is 0.405 ($P = 0.036$), meaning that moderate positive correlation between the results exists. Higher positive correlation exists between the FISH results and DNA concentration ($R = 0.629$, $P \leq 0.001$) than between the qPCR results and DNA concentration ($R = 0.553$, $P = 0.003$), even though DNA extraction is the pre-step for qPCR and not for FISH.

4.6. Cultivation

The growth of *F. prausnitzii* on different broths and agars is presented in Table 10. The cultivation of *F. prausnitzii* was challenging; the growth of the bacterium was extremely slow and the colonies on agar plates were small (Figure 13). When *F. prausnitzii* was transferred from one FAA-blood plate to several new ones, the bacterium grew on some of the new plates but not on all. The bacterium did not grow on FAA-blood plate after inoculation from FAB broth. In addition, the bacterium did not grow on FAA-blood plate after freezer storage. *F. prausnitzii* did not grow on any of the tested growth mediums in a basic anaerobic jar, indicating that the bacterium is an extremely strict anaerobe.

When cultivated on FAA-blood plates, *F. prausnitzii* DSM 17677 colonies were small and circular with full or undulated margin (Figure 13). The colonies were shiny and translucent, and the elevation was crateriform. No hemolysis was observed. In Gram-staining, *F. prausnitzii* was a Gram-negative, thin and twisted rod (Figure 14).

Table 10: The results of the *F. prausnitzii* cultivation study.

Medium	Growth	Medium	Growth
From DSM 17677 broth (from Germany)		**From FAB**	
FAA-blood	++	FAA-blood	-
CYE agar	-	FAA + Cb + Gluc	-
FAB	++	Wilkins-Chalgren agar	-
FAB + BHI	++	CYE agar	-
FAB + meat extract	+	FAB	++
BHI	+	FAB + Cb	++
BHI + meat extract	+	FAB + Gluc	++
Meat extract	-	FAB + Cb + Gluc	++
When DSM 17677 was cultivated on the same growth media as above but in a basic anaerobic jar instead of the anaerobic chamber, no growth could be inspected in any of the broths or agars.		BHI	-
		BHI + Cb	-
		BHI + Gluc	-
		BHI + Cb + Gluc	-
From FAA-blood plate		**From freezer (milk-glycerol solution)**	
FAA-blood	+	FAA-blood	-
FAA + Cb + Gluc	-	FAA + Cb + Gluc	-
Wilkins-Chalgren agar	-	Wilkins-Chalgren agar	-
CYE agar	-	FAB	+
FAB	++	FAB + Cb	+
FAB + Cb	++	FAB + Gluc	+
FAB + Gluc	++	FAB + Cb + Gluc	+
FAB + Cb + Gluc	++	BHI	-
BHI	-	BHI + Cb	-
BHI + Cb	-	BHI + Gluc	-
BHI + Gluc	-	BHI + Cb + Gluc	-
BHI + Cb + Gluc	-	Meat extract	-

The bacterial growth after inoculation from different growth environments is presented. The content of the original broth sent from DSMZ is not known. In general, the cultivation of the bacterium was very challenging. - = no growth; + = restricted growth; ++ = medium growth. All the broths and agars were made at the UTULab. Wilkings-Chalgren agar = Anaerobic agar (Oxoid ltd, England). BHI = Brain-Heart Infusion broth; CYE agar = legionella agar; Cb = 0.5 % w/v Cellobiose; Gluc = 0.5 % w/v Glucose.
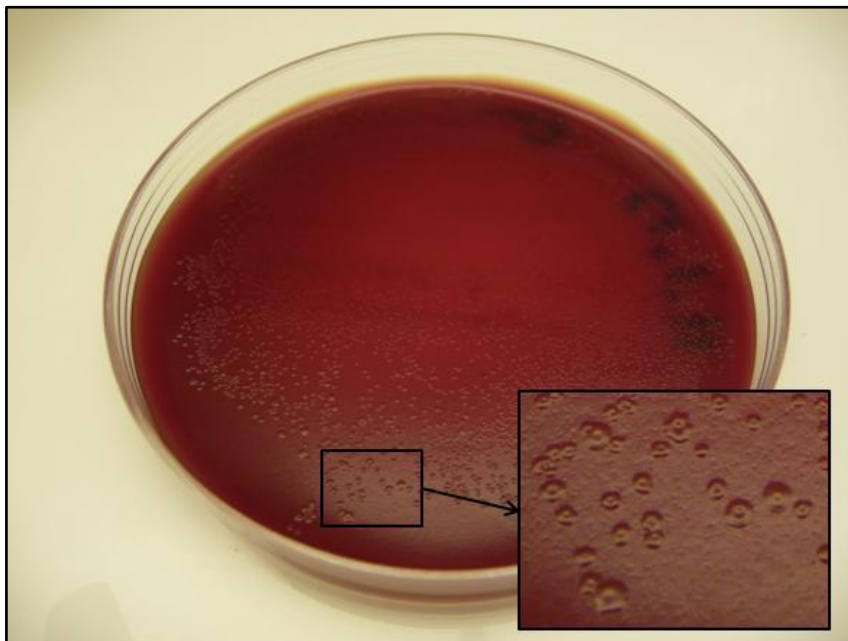

Figure 13: *F. prausnitzii* colonies on a FAA-blood plate. The colonies are small and circular, and the elevation is crateriform.
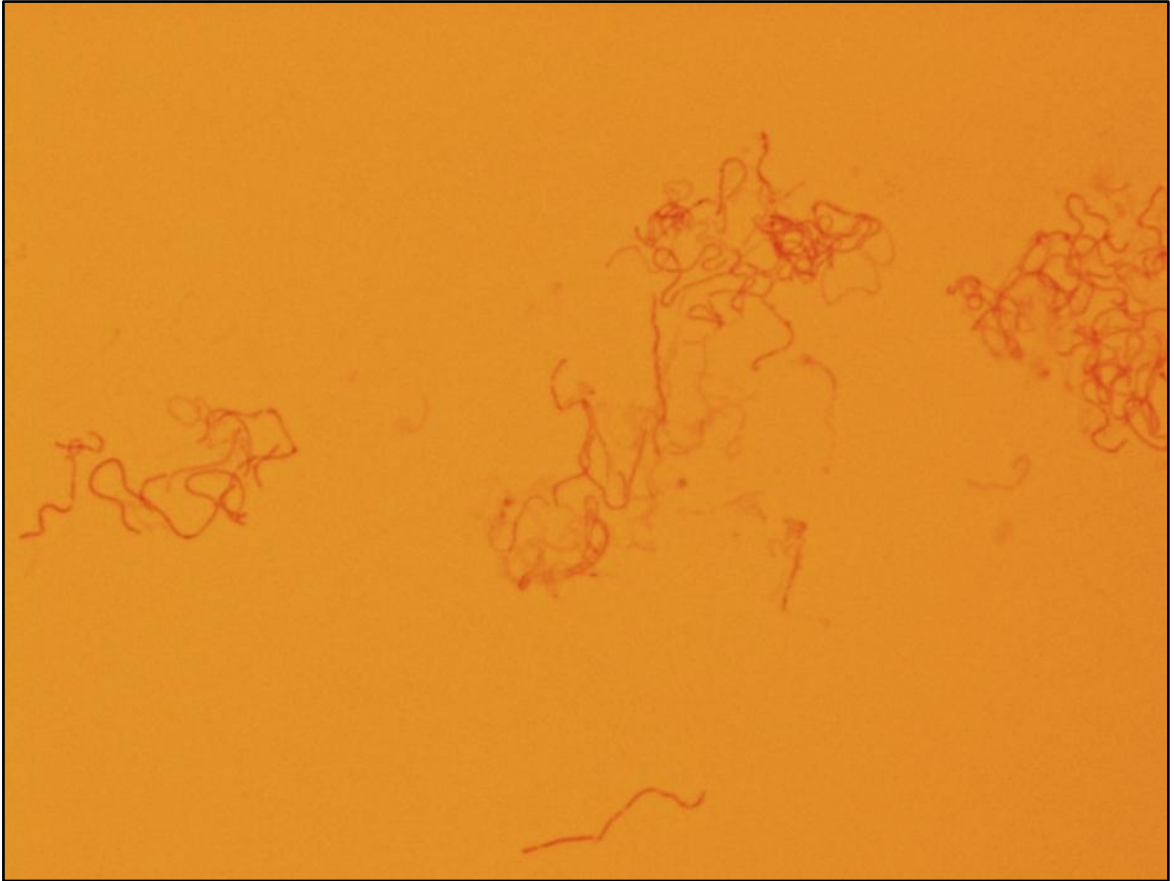
Figure 14: Gram-stained *F. prausnitzii*. The bacterium appears as a Gram-negative, thin and twisted rod.

5. DISCUSSION

The main purpose of the present study was to set up a practical quantitative method for investigating *F. prausnitzii* from fecal samples. The method is going to be used routinely in the UTULab clinical laboratory and in further research concentrating on gut bacteria. In this study, quantitative real-time PCR (qPCR) and fluorescence in situ hybridization (FISH) were compared to find out which of the two methods was most suitable for investigating *F. prausnitzii* from fecal samples. Both methods had their advantages and drawbacks, but in the end, qPCR was found much more practical for the routine quantification of *F. prausnitzii*.

The other main goal of this study was to cultivate *F. prausnitzii* and investigate its resistance to some of the most common antibiotics. The antibiotic resistance study could not be carried out during the time scheduled for this study, but successful cultivation of *F. prausnitzii* could be conducted.

5.1. The underestimated importance of DNA extraction

The first task of the present study was to validate an optimal DNA extraction method to be used in UTULab for extracting *F. prausnitzii* DNA from fecal samples. Different DNA extraction methods for human fecal samples have been previously compared, and the extraction method is known to strongly impact the amounts of the dominant bacteria found from fecal samples (Salonen et al., 2010, Maukonen et al. 2012; Nechvatal et al. 2007).

In this study, an enzyme based manual DNA extraction method (QIAamp DNA Stool Mini Kit, Qiagen, USA) was compared to a semiautomatic DNA extraction method (GXT Stool Extraction Kit, Hain Lifescience GmbH, Germany). The criteria for selecting the best DNA extraction method for the final validation were the gained total DNA quantity in relation to needed sample amount, the gained DNA quality, amount of gained *F. prausnitzii* DNA, the ease of use and hand-on-time, and the price of the method.

As can be seen from Tables 8 and 9 and from Figure 5, both the gained total DNA quantity and the *F. prausnitzii* 16S rRNA gene copy numbers were significantly higher in the

samples extracted with GXT. It is noteworthy that even the DNA gain with QIA + BB was significantly lower than with GXT performed without the extra homogenization (data not shown). The quality of the DNA was slightly better with QIA, especially the 260/230 ratios were significantly higher in some samples. A low 260/230 ratio may indicate the presence of contaminants which absorb at 230 nm, for example carbohydrates or phenol (T042 Technical Bulletin, Thermo Fisher Scientific – NanoDrop Products, USA). However, the DNA quality gained with either extraction method was sufficient for downstream applications without any extra purification, and the PCR procedures worked as expected.

The reproducibility of the extraction methods was compared by performing parallel extractions of two fecal slurries. The repeatability was better with GXT (Table 9), which was a bit expected, as in QIA the whole procedure was done manually while in GXT a robot did most of the pipetting. Freezing at -70˚C for one week did not affect the DNA quantity or quality of the samples, but did seem to slightly affect the reproducibility of both extraction methods (Table 9).

It has been previously noted that rigorous methods are most effective for the extraction of *Clostridium leptum* group and *F. prausnitzii* DNA (Maukonen et al. 2012; Salonen et al. 2010). This is why MO BIO homogenization was added to the extraction protocols. The mechanical lysis indeed seemed to increase both the DNA quantity and the *F. prausnitzii* 16S rRNA gene copy numbers (data not shown), which is in line with the previous results. Homogenization of five minutes (2 times 2.5 minutes) at 2000 rpm was chosen to be used in this study, because no notable increase in the DNA gain was seen after this certain time point when testing different homogenization times (data not shown).

Based on this study, of the two compared methods, GXT Stool Extraction Kit is recommended for bacterial DNA extraction from feces. The gained total DNA quantity was appreciably greater with GXT than with QIA ($P = 0.002$), and the *F. prausnitzii* 16S rRNA gene copy number was also significantly greater in the samples extracted with GXT ($P = 0.003$). The DNA quality was better with QIA, but also the quality gained with GXT was sufficient for downstream applications. In addition, the simplicity and cost effectiveness of the GXT method overcame QIA. It is noteworthy though, that both the

DNA quantity and quality differed notably between each fecal sample. This is understandable, because the composition of each fecal sample is different, and for this reason, neither of the tested extraction methods is perfect for every fecal sample.

QIAamp DNA Stool Mini Kit is widely used in molecular research purposes and several published study results rely on this particular extraction method, even though its maintenance has been questioned by several research groups (Maukonen et al., 2012; Salonen et al., 2010). Based on the results of this present study, the qPCR results gained after QIA extraction should not be trusted due to severe deficiency in the gained total DNA quantity.

5.2. Detection of *F. prausnitzii* abundance

Before setting up the quantitative PCR, traditional PCR was used to test the function of the chosen PCR primers and PCR conditions. Traditional PCR with primers Fprau 07 and Fprau 02 worked well under the chosen reaction conditions; all the fecal samples gave a band in the AGE run at the expected size ~140 bp (Figure 4), indicating that all the studied samples included at least some amount of *F. prausnitzii* DNA. The intensities of the bands varied between the samples, indicating variations in either the *F. prausnitzii* abundances or in the PCR efficiency. Negative controls did not usually give any bands in traditional PCR, indicating that the primers did not bind to the tested control strains and that no contaminations occurred during the PCR procedure. Once, however, the negative controls gave dim bands, but the reason was just a minor contamination during the pipetting and the problem was fixed by simply repeating the PCR.

Both qPCR and FISH confirmed the results gained with the traditional PCR; all of the studied fecal samples included at least some amount of *F. prausnitzii*, and the abundances differed notably between samples.

5.2.1. Drawbacks of qPCR

One main drawback of qPCR is the lack of actual *F. prausnitzii* –quantification. 16S rRNA gene copy numbers cannot be directly converted into cell counts, because the number of the 16S rRNA gene copies varies among bacterial species (Lee et al., 2009). However, the

gene copy numbers can still be considered to be good indicators of *F. prausnitzii* proportions in fecal samples.

The gained *F. prausnitzii* 16S rRNA gene copy numbers in this present study (Figure 6) were in line with the results gained from healthy subjects in a previous study by Sokol and co-workers (2009). It is noteworthy though, that a notable difference in the gene copy numbers of samples FP4, FP16, FP17 and FP18 can be seen between the results represented in Figure 5 and Figure 6. This difference is most probably a consequence of 1:100 sample dilutions being used for the final study cohort instead of 1:500, which was used in the previous qPCR runs. The efficiency of *F. prausnitzii* PCR thus seems to be higher with the more diluted samples. The correlation between the results in Figures 5 and 6 is very strong ($R = 0.988$; $P = 0.000$) meaning that the relative *F. prausnitzii* abundances between the samples are equal in both qPCR runs. Because the effect of the sample dilution on qPCR results was not further studied, the gained results are not completely trustworthy.

One possible source of error in the qPCR of this study was the gained DNA quality. Even though the DNA extracted with GXT seemed to suffice well for the PCR analysis, it cannot be guaranteed that there were no PCR inhibitors that affected the results. This should have been ensured by purifying part of the GXT-extracted DNAs and repeating the qPCR run for both the unpurified and purified DNA.

5.2.2. qPCR and sequencing data revealed a problem in the *F. prausnitzii* primers

When comparing the sequences of DSM 17677 and the DNA standard pool made from positive PCR products (Figure 8), it can be noted that the sequences are otherwise identical, but the pooled DNA includes an uncertain area "RY". In sequence analysis, R means purine (G or A), and Y means pyrimidine (either C or T). When comparing the pooled DNA sequence to the *F. prausnitzii* 16S rRNA gene sequences found from BLAST database (National Center for Biotechnology Information), it was noted that some of the *F. prausnitzii* strains include "GC" and some include "AT" at this particular sequence site. This means that heterogeneity exists between different *F. prausnitzii* strains in the short

DNA fragment amplified with primers Fprau 07 and Fprau 02. The "RY" in the pooled DNA sequence thus implicates that both sequence types exist in the DNA pool.

The heterogeneity between the different *F. prausnitzii* strains explains the two main populations and the skewed peaks in the qPCR melt curve analysis (Figure 7) that is based on the heat-denaturation of the double-stranded PCR products. In double-stranded DNA, the DNA strands are held together by hydrogen bonding between the complementary nucleotides; cytosine and guanine are held together by three hydrogen bonds while adenine and thymine are held together by two hydrogen bonds. The A-T bond is thus slightly weaker than the G-C bond, and the differences in the DNA sequences can be seen in the melt curve analysis because G-C bonds can hold together in slightly higher temperatures.

The melting temperature peak of DSM 17677 was sharper than the DNA standard pool melting peak (Figure 7), because the qPCR of the standard pool led to two products with different denaturing temperatures. The melt curve of the DNA standard pool indicates that "AT" was the predominant sequence in the uncertain area, because the average melting temperature of the pool was lower than the melting temperature of DSM 17677.

The melt curves of the studied fecal samples were mostly situated somewhere between DSM 17677 and the DNA standard pool (Figure 7), indicating that most of the samples included both of the two sequence types in various relative abundances. Some of the samples had sharp melt peaks and thus seemed to include only one type of product, though.

To overcome the problem of two products in qPCR, new PCR primers should be designed so that the amplified DNA fragment does not include heterogeneity between different *F. prausnitzii* strains. The primer design was not included in this study, but has to be executed before further studies.

5.2.3. qPCR rises above FISH in easiness and reproducibility

In this present study, the relative *F. prausnitzii* proportion of the fecal samples gained with FISH varied from 1 % to 46 % (Figure 12, Appendix II). The result range was larger than in the previous studies (Suau et al. 2001; Mueller et al. 2006), in which the *F. prausnitzii*

percentages have varied between 5 % and 28 %. The total bacterial counts gained with FISH (Figure 12) were perfectly in line with the results of a previous study by Harmsen and co-workers (1999).

The morphology of *F. prausnitzii* was very different in fixed DSM 17677 samples than in the fixed fecal samples (Figures 9 and 10). This was most probably a consequence of the freezer storage of the fecal samples before performing the cell fixation. The *F. prausnitzii* morphology of the fecal samples (Figure 11) was in line with the observations of Suau and co-workers (2001), who also noted that *F. prausnitzii* often appeared in pairs. They had called the dominant morphology "asymmetrical double droplets".

When compared to qPCR, microscopy-FISH was a very time-consuming and laborious analysis method. The cell fixation and hybridization both required an overnight incubation, and the microscopy analysis of the samples was very time-consuming. It thus took at least three days to get any results with FISH, while with qPCR the results could be obtained in one day. Additionally, the counting of the bacterial cells with the microscope was not straightforward; the fluorescent dyes worked a bit differently in every sample, and the cell number varied enormously between each field of vision due to the uneven distribution of the bacteria into the observed filters. For this reason, the reproducibility of qPCR was found to be much better.

The Flow cytometry –FISH enables the analysis of bacteria more rapidly and more accurately than microscopy (Vaahtovuo et al., 2005), but of course this would not dispense from the overnight incubations of the sample preparation process. Flow cytometry –FISH could still be a considerable option to qPCR, since it would not include the DNA extraction step that may significantly underestimate the number of certain bacterial species.

The qPCR and FISH results of this present study are not completely reliable. The sample number was very small ($n = 27$) and thus does not qualify for a reliable quantitative research. The DNA extractions and the fixations of the fecal samples were only performed once due to limited sample amounts. The qPCR runs were performed several times for most of the samples, but the FISH protocol was repeated only if the results differed notably

from the expected ones. For more reliable results, FISH should have been repeated at least once for all the samples. However, the repeatability of the analyses is good due to precise notes and instructions, which increases the reliability of the results of this study.

Only moderate positive correlation between the gained FISH and qPCR results was observed ($R = 0.405$, $P = 0.036$), and for some samples the results were extremely different (Appendix II) meaning that either of the methods led to false or undetermined results. Due to much better reproducibility, qPCR results can be considered more trustworthy.

Some clinical characteristics of the study group, such as body mass index, fat percentage and blood triglyceride level, were introductorily compared to the gained FISH and qPCR results, and especially the relative *F. prausnitzii* percentages gained with FISH correlated positively with several parameters related to metabolic syndrome (data not shown). Due to such a small study cohort, no reliable statistical analyses could be done and thus the analysis of the clinical data was entirely excluded from this study. However, the interesting preliminary results boost the interest to further investigate the role of *F. prausnitzii* in human health.

5.3. Results of the cultivation study

In advance, the *F. prausnitzii* cultivation was considered a serious task, because the bacterium in question is a strictly obligatory anaerobe that does not tolerate any oxygen (Moore and Holdeman, 1974). Indeed, the cultivation of the bacterium was found to be quite challenging and could be performed only in an anaerobic chamber (Table 10).

Even though *F. prausnitzii* grew on FAA-blood plates and in FAB, the mediums obviously did not provide the optimal growth environment for the bacterium; the growing was very slow and the colonies on agar plates were very small (Figure 13). It was also witnessed that when *F. prausnitzii* was inoculated from one FAA-blood plate to several new ones, the bacterium grew on some of the new plates but not on all. For this reason, the inoculation of the bacterium had to be done always on several plates at once.

The main reason for cultivating *F. prausnitzii* was the idea of performing antibiotic resistance tests on agar plates. Plate cultivation enables simple reading of the results and excludes the risk of unnoticeable contaminations. Another idea was to develop a selective agar plate that could be used for isolating *F. prausnitzii* from fecal samples. Due to the very challenging cultivation of the bacterium, neither of these goals could be fulfilled during the limited time scheduled for this study. If the resistance testing is performed in the future, it would probably be best to conduct in broths instead of agar plates, because in broths, the growth of the bacterium was more likely. The isolation of *F. prausnitzii* from fecal samples by plating will also be a real challenge due to the strict growth requirements of the bacterium.

In Gram-staining, the morphology of *F. prausnitzii* was quite distinctive (Figure 14). The bacterium seemed to be Gram-negative, and the cells looked like thin and long spirally rods. It is presumable though, that the long rods consisted of several *F. prausnitzii* cells. Quite few single cells were seen using microscopy, suggesting the tendency of the *F. prausnitzii* cells to aggregate. Yet it is uncertain if the seen morphology actually is characteristic of *F. prausnitzii*, or if it resulted from the growth habitat not being optimal.

5.3.1 Antibiotic resistance of *F. prausnitzii* – an interesting future research topic

The antibiotic resistance of commensal gut bacteria has not gained much interest on the research field, even though antibiotics can lead to severe changes in the gut microbiota. Antibiotics are also commonly used in treatment of IBD, even though little is known about the effects of antibiotics on gut microbiota (Swidsinski et al., 2008).

It is proposed that the fecal number of *F. prausnitzii* is lowered due to long treatment periods with antibiotics (Bartosch et al., 2004), but the antibiotic resistance of *F. prausnitzii* has not in our knowledge been studied previously. Kaye and co-workers (1980) have tested the antibiotic susceptibility of anaerobic gut bacteria in general – in their research, one *F. prausnitzii* strain was included in the *Fusobacterium sp.* group, but no results of *F. prausnitzii* as an individual were released. A tetracycline resistance gene tet(W) has been found in *F. prausnitzii* strains isolated from human feces by Scott and co-

workers (2000). Other publications related to *F. prausnitzii* antibiotic resistance could not be found, so to our knowledge, the antibiotic resistance of different *F. prausnitzii* strains has not been further tested.

If *F. prausnitzii* had notable impact on gut homeostasis and the susceptibility of *F. prausnitzii* to different antibiotics varied, this could be the key between antibiotic usage and some gut disorders. Though, it is likely that the conditions are much more complicated. In any case, the antibiotic resistance analysis for *F. prausnitzii* is an interesting topic for future research.

5.4. Summary

The general purpose of this study was to develop protocols for practical quantification of *F. prausnitzii* from fecal samples. The protocols developed in this pilot study will be exploited in future research regarding the clinical significance and possible health marker status of *F. prausnitzii*. Several goals of this study were achieved, and some extremely beneficial results were gained. This study proved that GXT Stool Extraction Kit combined with MO BIO homogenization is an effective method for bacterial DNA extraction from feces; thus the method in question can be recommended to be used in future research. In addition, it was proven that qPCR is an effective method for the quantitative analysis of *F. prausnitzii* from stool. The *F. prausnitzii* qPCR protocol must be revised before future studies, but the validated methods can then relatively easily be modified for other bacterial species too. Also the cultivation of *F. prausnitzii* can be explored in future studies, but needs some intensive development to be suitable for the isolation of *F. prausnitzii* from feces and for the antibiotic resistance testing.

Altogether, the techniques developed in this study can be used as basic methods in the forthcoming gut microbiota studies conducted at the Department of Medical Microbiology and Immunology, University of Turku, and they can also be utilized in routine diagnostics performed in UTULab.

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Preparation of some of the most essential solutions used in this study

4 % Paraformaldehyde for cell fixation

Recipe for one fecal sample; all reagent quantities shall be multiplied by the number of samples to be fixed. The prepared solution is usable for three days.

- Weight 0.4 g 95 % paraformaldehyde powder (e.g. Sigma-Aldrich Co., USA)
- Add 2 ml H_2O and 20 µl 1 M NaOH
- Heat ~60˚C and mix occasionally till paraformaldehyde has fully dissolved
- Allow the solution to cool for a while, and then add 8 ml PBS

1M Tris-HCl for hybridization solution

- Weight 60.57 g Trizma®base (Sigma-Aldrich Co., USA)
- Add 400 ml H_2O, mix to dissolve
- Add carefully 35 ml 37.2 % HCl
- Mix and allow to cool
- Check the pH, adjust to ~7.2
- Add H_2O ad. 500 ml

Hybridization solution with 5 % formamide

- Weight 5.26 g NaCl → final concentration 0.9 M
- Add H_2O till NaCl is dissolved
- Add 2 ml 1M Tris-HCl → final concentration 20 mM
- Add 2 ml 5 % SDS → final concentration 0.1 %
- Add 5 ml 99.5 % formamide (Acros Organics, Belgium)
- Add H_2O ad. 100 ml

DAPI wash solution

- Weight 13.15 g NaCl → final concentration 0.9 M
- Add H_2O till dissolved
- Add 5 ml 1 M Tris-HCl → final concentration 20 mM
- Add H_2O ad. 250 ml
- Add 125 µl DAPI stock 1 mg/ml
- Storage at dark

5% Chelex®

- Weight 5 g Chelex® 100 Resin (Bio-Rad Laboratories, USA)
- Add H_2O ad. 100 ml

DNA quantities, FISH results and qPCR results of the final study cohort

As FISH results, both the *F. prausnitzii* percentages and the *F. prausnitzii* cell numbers per one gram of feces (wet weight) are represented. As qPCR results, *F. prausnitzii* 16S rRNA gene copy numbers per one gram of feces (wet weight) are represented.

Sample code	DNA conc. (ng/µl)	260/280	260/230	F. prausnitzii % (FISH)	F. prausnitzii cell number (FISH)	F. prausnitzii 16S rRNA gene copy number
FP4c	236.8	1.83	1.42	13.48 %	1.89E+10	1.95E+11
FP5	75.8	1.94	1.51	11.45 %	4.68E+09	5.17E+10
FP16a	134.5	1.82	1.21	8.93 %	5.65E+09	4.24E+10
FP16b	286.1	1.81	1.55	1.52 %	2.20E+09	7.84E+10
FP17a	176.9	1.94	1.60	16.12 %	1.50E+10	2.82E+10
FP17b	304.1	1.89	1.47	17.03 %	1.41E+10	4.88E+10
FP18a	579.7	1.74	1.04	37.00 %	4.31E+10	2.96E+11
FP18b	332.6	1.72	1.08	10.25 %	1.47E+10	1.54E+11
FP20	131.8	1.93	1.43	46.31 %	1.60E+10	1.31E+11
FP21	460.3	1.75	0.96	22.58 %	1.86E+10	4.38E+11
FP22	444.3	1.84	1.40	25.59 %	1.05E+10	7.67E+10
FP23	216.4	1.83	1.26	9.86 %	1.35E+10	1.21E+11
FP24	496	1.91	1.70	28.71 %	4.22E+10	9.74E+10
FP25	280	1.75	1.36	7.80 %	6.06E+09	2.61E+11
FP26	281.1	1.99	1.67	3.34 %	8.27E+08	6.68E+10
FP27	375.5	1.83	1.44	8.21 %	5.24E+09	6.53E+10
FP28	312.6	1.7	1.17	5.22 %	6.48E+09	2.70E+11
FP29	441.2	1.92	1.76	28.57 %	7.44E+09	1.38E+11
FP32*	355.1	1.81	1.38	25.71 %	1.36E+10	7.46E+10
FP34	122.7	1.98	1.72	10.23 %	1.24E+09	8.29E+09
FP35	370.7	1.99	1.82	40.49 %	3.84E+10	9.52E+10
FP36	287.5	1.93	1.58	24.74 %	1.65E+10	7.48E+10
FP37	352.7	1.74	1.19	27.35 %	4.22E+10	2.20E+11
FP38	209.7	1.98	1.62	11.54 %	1.03E+10	8.29E+09
FP39	489.7	1.79	1.47	19.93 %	4.18E+10	2.08E+11
FP40	191.0	1.66	0.80	24.87 %	9.65E+09	7.04E+10
FP41	79.5	2.02	1.84	1.08 %	5.51E+08	1.27E+10

*The extracted DNA-solution included black magnetic particles, meaning that the extraction protocol with GXT had not worked properly