REAL-TIME PCR – A Molecular Approach to Investigate the Role of Intestinal Microbiota in the Pathophysiology of Irritable Bowel Syndrome

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ACADEMIC DISSERTATION

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Helsinki 2011
This thesis is dedicated to the memory
of my late parents, Aune and Esko
# TABLE OF CONTENTS

## ABSTRACT

## LIST OF ORIGINAL PUBLICATIONS

## ABBREVIATIONS

### 1. INTRODUCTION

### 2. REVIEW OF THE LITERATURE

### 2.1 Human intestinal microbiota

#### 2.1.1 Establishment of the intestinal microbiota

#### 2.1.2 Composition of the adult GI microbiota

#### 2.1.3 Functions of the gut microbiota

#### 2.1.4 Role of gut microbiota in disease

#### 2.1.5 Role of intestinal microbiota against enteric pathogens

### 2.2 Culture-independent techniques for microbiota characterization

#### 2.2.1 Characteristics of 16S rRNA gene

#### 2.2.2 Sequencing analysis of 16S ribosomal DNA populations

#### 2.2.3 Percent guanine + cytosine profiling

#### 2.2.4 Quantitative real-time PCR

### 2.3 Irritable bowel syndrome

#### 2.3.1 Definition and description of the disease

#### 2.3.2 Diagnosis of IBS

#### 2.3.3 IBS subtypes

#### 2.3.4 Pathophysiological mechanisms and etiological factors in IBS

#### 2.3.5 Intestinal microbiota and IBS

#### 2.3.6 Therapeutic modulation of intestinal microbiota in IBS

### 3. AIMS OF THE STUDY

### 4. MATERIALS AND METHODS

#### 4.1 Study subjects

##### 4.1.1 IBS patients (I-IV)

##### 4.1.2 Healthy control subjects (I, II, IV)

#### 4.2 Study design of the probiotic intervention

#### 4.3 Collection of fecal samples

#### 4.4 Intestinal microbiota analysis of IBS patients and controls

#### 4.5 Molecular microbiological and biochemical techniques

##### 4.5.1 DNA extraction for real-time PCR analyses (I-IV)

##### 4.5.2 Design of real-time PCR assays (I, IV)

##### 4.5.3 Assay optimization (I, IV) and amplification conditions (I-IV)

##### 4.5.4 Preparation of genomic DNA standards (I-IV)

##### 4.5.5 Real-time PCR data interpretation (I-IV)

##### 4.5.6 Sequencing of selected PCR products (II, IV)

##### 4.5.7 Analysis of selected intestinal metabolites (III)

#### 4.6 Statistical analysis

#### 4.7 Ethical issues
5. RESULTS AND DISCUSSION ................................................................. 44
   5.1 General optimization of SYBR Green I-based PCR assays (I, IV) .................. 44
      5.1.1 Sensitivity and linearity of the assays ............................................. 44
      5.1.2 Specificity of the assays ................................................................. 44
   5.2 Applicability of real-time PCR for fecal bacterial analysis (I) .................... 45
   5.3 Intestinal microbiota in IBS patients and healthy controls (II) .................. 47
   5.4 Effect of probiotic supplementation on the gut microbiota in IBS patients (III) .... 48
   5.5 Screening of pathogens in fecal samples of IBS patients and healthy controls (IV) .... 51
6. CONCLUDING REMARKS .................................................................... 54
7. ACKNOWLEDGEMENTS ..................................................................... 56
8. REFERENCES ...................................................................................... 57
ABSTRACT

Irritable bowel syndrome (IBS) is a common multifactorial functional intestinal disorder, the pathogenesis of which is not completely understood. Increasing scientific evidence suggests that microbes are involved in the onset and maintenance of IBS symptoms. The microbiota of the human gastrointestinal (GI) tract constitutes a massive and complex ecosystem consisting mainly of obligate anaerobic microorganisms making the use of culture-based methods demanding and prone to misinterpretation. To overcome these drawbacks, an extensive panel of species- and group-specific assays for an accurate quantification of bacteria from fecal samples with real-time PCR was developed, optimized, and validated. As a result, the target bacteria were detectable at a minimum concentration range of approximately $10^4$ bacterial genomes per gram of fecal sample, which corresponds to the sensitivity to detect 0.000001% subpopulations of the total fecal microbiota.

The real-time PCR panel covering both commensal and pathogenic microorganisms was assessed to compare the intestinal microbiota of patients suffering from IBS with a healthy control group devoid of GI symptoms. Both the IBS and control groups showed considerable individual variation in gut microbiota composition. Sorting of the IBS patients according to the symptom subtypes (diarrhea, constipation, and alternating predominant type) revealed that lower amounts of *Lactobacillus* spp. were present in the samples of diarrhea predominant IBS patients, whereas constipation predominant IBS patients carried increased amounts of *Veillonella* spp. In the screening of intestinal pathogens, 17% of IBS samples tested positive for *Staphylococcus aureus*, whereas no positive cases were discovered among healthy controls.

Furthermore, the methodology was applied to monitor the effects of a multispecies probiotic supplementation on GI microbiota of IBS sufferers. In the placebo-controlled double-blind probiotic intervention trial of IBS patients, each supplemented probiotic strain was detected in fecal samples. Intestinal microbiota remained stable during the trial, except for *Bifidobacterium* spp., which increased in the placebo group and decreased in the probiotic group.

The combination of assays developed and applied in this thesis has an overall coverage of 300-400 known bacterial species, along with the number of yet unknown phylotypes. Hence, it provides good means for studying the intestinal microbiota, irrespective of the intestinal condition and health status. In particular, it allows screening and identification of microbes putatively associated with IBS. The alterations in the gut microbiota discovered here support the hypothesis that microbes are likely to contribute to the pathophysiology of IBS. The central question is whether the microbiota changes described represent the cause for, rather than the effect of, disturbed gut physiology. Therefore, more studies are needed to determine the role and importance of individual microbial species or groups in IBS. In addition, it is essential that the microbial alterations observed in this study will be confirmed using a larger set of IBS samples of different subtypes, preferably from various geographical locations.
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):


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**ABBREVIATIONS**

AAD  antibiotic-associated diarrhea  
A-T  adenine-thymine  
bp  base pair  
CFU  colony-forming unit  
CD  Crohn’s disease  
CI  confidence interval  
C<sub>r</sub>  Threshold cycle  
DGGE  denaturing gradient gel electrophoresis  
DNA  deoxyribonucleic acid  
dsDNA  double-stranded deoxyribonucleic acid  
EBI  European Bioinformatics Institute  
FISH  fluorescent in situ hybridization  
FOS  fructo-oligosaccharide  
FRET  fluorescence resonance energy transfer  
G+C  guanine plus cytosine  
GI  gastrointestinal  
HITChip  Human Intestinal Tract Chip  
IBD  inflammatory bowel disease  
IBS  irritable bowel syndrome  
IBS-C  constipation-predominant IBS  
IBS-D  diarrhoea-predominant IBS  
IBS-M  mixed-subtype IBS  
%ID  percentage identity  
IL  interleukin  
kb  kilobase  
Mb  megabase  
NCBI  National Center for Biotechnology Information  
NTC  no-template control  
ob/ob  genetically obese  
PAR-2  proteinase-activated receptor two  
PI-IBS  post-infectious IBS  
qPCR  quantitative real-time polymerase chain reaction  
RDP II  Ribosomal Database Project II  
Redox  oxidation-reduction  
rRNA  ribosomal ribonucleic acid  
SCFA  short-chain fatty acid  
SIBO  small intestinal bacterial overgrowth  
SRB  sulfate-reducing bacteria  
T<sub>a</sub>  annealing temperature  
TGGE  temperature gradient gel electrophoresis  
T<sub>m</sub>  melting temperature  
TNF-α  tumor necrosis factor-alpha  
TRAC  transcript analysis with aid of affinity capture  
T-RFLP  terminal restriction fragment length polymorphism  
UC  ulcerative colitis  
UV  ultraviolet  
λ<sub>max</sub>  maximum wavelength
1. INTRODUCTION

Humans have been associated with complex microbial communities throughout their evolution. The microorganisms inhabiting the skin, mucosa, and alimentary tract of an adult outnumber the human cells by a factor of 10 to 100 (132). The vast majority of gastrointestinal (GI) tract bacteria reside in the colon, where densities approach \(10^{11}-10^{12}\) cells/g, the highest recorded for any microbial habitat (15, 215, 330). The gut microbiota therefore extends the human genome such that humans can be described as human-microbial superorganisms (155). Since today there are approximately 6.5 billion humans living on Earth, this represents a gut reservoir of \(10^{23}-10^{24}\) microbial cells. Thus, the human GI tract represents a considerable microbial habitat in the biosphere, which is likely to reflect the co-evolution between the microbial community and its host (330).

More than 400 different bacterial species have been isolated and characterized from human GI tract samples (346). However, the cultivable intestinal bacteria represent only a fraction of the microbes actually present in the gut. Recent data incorporating the information obtained with modern culture-independent molecular methods have revealed that the diversity of the GI tract microbial community is significantly higher than anticipated (40, 97, 250, 292, 301, 314). By integrating the results from cultivation-based studies with recent data derived largely from the 16S rRNA gene sequencing studies, it has been estimated that the human GI tract harbors more than 1000 distinct microbial species (250). However, this number is likely to increase as further research data become available. A relatively large proportion of the newly discovered microorganisms are as yet undescribed or not recovered, and have little relatedness to any known bacterial species or even genus (87, 123, 130). Importantly, the intestinal community structure is currently being extensively characterized by several research groups in order to obtain a more comprehensive picture of the microbiota. Much work remains to establish the causes and consequences of temporal and inter-individual variation in microbiota community composition and to understand the cellular basis of GI tract host-microbe interactions (74, 168, 347).

Irritable bowel syndrome (IBS) is a common multifactorial functional intestinal disorder of unknown etiology (174). It is considered a major cause of abdominal discomfort and gut dysfunction worldwide with an estimated prevalence of 10% to 20% of the adult population, which makes it the most frequent diagnosis in gastroenterology (174, 209, 259). Although not life-threatening, IBS is a major global health problem resulting in significant sensation of illness, poor quality of life, a high rate of work absenteeism and considerable health costs (77, 284). IBS is characterized by a variable combination of chronic and recurrent symptoms including abdominal pain or discomfort, irregular bowel movements, flatulence, and constipation or diarrhea (306). According to the stool consistency, IBS subjects can be divided into three subcategories predominant in diarrhea (IBS-D), constipation (IBS-C) or alternating constipation and diarrhea i.e., the mixed subtype (IBS-M) (77, 174, 306).

The mechanisms of pathogenesis behind IBS are only partly understood and thus cannot be traced to a single organic factor. Instead, IBS is considered a complex biopsychosocial condition in which a multitude of mechanisms at the central and peripheral level interact (80). The proposed mechanisms contributing to the etiology of IBS symptoms include visceral hypersensitivity, abnormal motor function, low-grade mucosal
Introduction

inflammation, food intolerance, altered gut microbiota as well as psychosocial and genetic factors (10, 19, 81). However, it is often difficult to differentiate between the causes and effects, especially for chronic impaired states of health (286).

Quantitative real-time polymerase chain reaction (qPCR) is a powerful advancement of the conventional end-point PCR, which enables quantification of the target nucleic acid from different matrices and reduces significantly the risk of ‘carry-over’ contamination (126). Generally, the technique has exceptional performance characteristics including a wide dynamic range (up to 7 orders of magnitude) and high sensitivity. Therefore, it is a superior method for the accurate and targeted quantification of specific bacterial species or groups within microbial populations. The purpose of the present study was to develop real-time PCR-based applications for the analysis of fecal bacterial populations, in order to uncover putative alterations in the intestinal microbial community structure of people suffering from IBS.
2. REVIEW OF THE LITERATURE

2.1 HUMAN INTESTINAL MICROBIOTA

2.1.1 Establishment of the intestinal microbiota

Because the conditions in the in utero environment are germ-free, the microbes that colonize the infant GI tract must come from the outside. During and after birth, infants are exposed to microbes that originate from the maternal birth canal and the surrounding environment. This explains the remarkable inter-individual variability observed in the infant GI tract microbiota during early life (233, 313). The GI tract is initially colonized by facultative aerobes such as Enterobacteriaceae, Enterococcus, and Streptococcus, since the intestinal environment of neonates shows a positive oxidation/reduction potential at birth. However, oxygen consumption by these bacteria alters the gut environment to more reducing conditions, which facilitates subsequent growth and colonization of the obligate anaerobes, including bifidobacteria, Bacteroides, Clostridium, and Ruminococcus (33, 92, 183, 216, 233).

Mode of delivery has frequently been cited as one of the key factors influencing the infant GI tract microbial community structure. The GI microbiota of infants delivered by cesarean section has been reported to encompass lower total bacterial counts, especially Escherichia coli, bifidobacteria, and Bacteroides, in early weeks of life (90, 109, 117, 238). This is due to the fact that delivery by cesarean section prevents the newborn from being exposed to bacteria in the birth canal (279). There are thus indications that intestinal colonization is delayed in infants delivered by cesarean section, and that the changes in intestinal microbiota can be persistent (109).

The reports regarding the effect of diet on the composition of the infant GI tract microbiota have been conflicting. Several independent studies have reported a lower abundance of bifidobacteria or a higher abundance of aerobic bacteria in the gut microbiota of formula-fed infants relative to breast-fed infants (93, 122, 134), but other reports have found no such difference (176, 238). One possible reason for the similarity of the diet-derived effects could be improved knowledge of the repertoire of human milk oligosaccharides, and their inclusion in commercial infant formula (89).

The second major change in intestinal microbial community composition occurs at weaning and introduction to a solid, adult-like diet (93). At this stage, the initially chaotic and unstable microbiota is enriched by bacteria that are common in the adult GI tract, such as Bacteroidetes and Firmicutes, as well as common occurrence of Verrucomicrobia, thus overcoming the initial advantage of facultative early-colonizing microbes that are less well adapted to the intestinal environment. After this, only relatively small changes take place and the microbiota stabilizes towards that of a stereotypical adult-like community, usually after one year of age (233).

2.1.2 Composition of the adult GI microbiota

The human body could be considered a metacommunity comprising many local microbial communities in different ecological niches. Each individual anatomical site has its unique physiochemical characteristics and each location is inhabited by a specialized microbial composition (165). The vast majority of the microorganisms are found in the intestinal tract, where microbial abundance and diversity increase from the stomach to the colon (266). The human GI tract contains
all three domains of life — **Bacteria, Archaea,** and **Eukarya.** Bacteria are undoubtedly predominant, but archaea, yeasts, fungi, and protozoa are also part of the intestinal microbiota (212, 266, 268).

Although the microbial diversity on Earth is enormous (e.g., 55 divisions of **Bacteria**), the human intestine is remarkable for its exclusivity. Once the microbiota of the infant gut has stabilized and converged towards a more adult community structure, it is dominated by members of just three divisions of bacteria — the **Bacteroidetes, Firmicutes,** and **Actinobacteria** (4, 87, 97, 157, 169, 292, 338). The prevalence of over 80% of these bacterial phyla in the GI tract has been reported in several large-scale 16S rRNA gene sequence surveys (4, 87, 157, 301). However, regardless of the uniformity in the phylum level distribution, the same studies showed a significant inter-individual variation in the species level diversity of the gut microbiota. It is likely that this variation arises from the accumulated effects of genetic and environmental influences on the gut microbial community (74).

**Microbiota of the upper GI tract**

Considerable microbial exposure derives from the diet, which is of major importance as a reservoir of microorganisms and a substrate to the intestinal microbes. Mechanical and enzymatic digestion of food initiates in the mouth and continues in the stomach. Due to its harsh acid environment (pH 1-2) and digestive enzymes such as pepsin, levels below $10^3$ bacteria per ml are commonly present in the gastric fluid (276). Thus, gastric juice is one of the body’s most effective defenses against pathogenic microorganisms. However, certain microbial species are able to remain viable in the stomach at low population levels. The most renowned and undisputed bacterium associated with the gastric mucosa in some individuals is **Helicobacter pylori,** which plays a role in development of human gastritis, peptic ulcer disease, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma (293). In contrast, relatively little is known about the presence of other bacterial species in the stomach. In a 16S rRNA gene survey, Bik and co-workers (35) discovered a diverse gastric microbial community which, in addition to **H. pylori,** comprised 128 bacterial phylotypes. The majority of the sequences were assigned to the **Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes,** and **Fusobacteria** phyla. Furthermore, Andersson et al. (4) revealed 262 phylotypes, representing 13 phyla, in **H. pylori-negative** gastric biopsy samples. These included sequences from phyla not detected in the stomach previously, e.g., **Chlamydia** and **Cyanobacteria.** Surprisingly, the samples that tested positive for **H. pylori** in culturing were completely dominated by this species (93-97% of the sequences), thus dramatically reducing the diversity. These observations indicate how well **H. pylori** is adapted to the gastric environment. In both studies, a large degree of inter-subject variability in the absence of **H. pylori** was observed, even for the most dominant phylotypes (4).

The small intestine, which consists of the duodenum, jejunum, and ileum, is the GI tract compartment where most of the digestion and absorption of nutrients occurs. As the contents of the stomach become liquefied, they pass into the duodenum. Due to the low pH and rapid passage of intestinal contents, the duodenal bacterial counts are low (approximately $10^4$ cells per ml of digesta). Subsequently, digesta flows from the duodenum through the jejunum to the end of the ileum within 4 hours, during which it has been converted to a neutral-alkaline pH 8 (151). As a consequence, the bacterial numbers increase up to $10^8$ cells per ml of digesta in the distal ileum (43, 124). The microbial community structure and
population dynamics in the small intestine remain largely unknown, primarily due to the challenges in sampling these locations.

Current knowledge regarding the small intestine microbiota derives mainly from biopsy samples collected during colonoscopy, colonic resection, ileal effluent, or from sudden death victims (27, 43, 124, 328, 335). According to these 16S rRNA gene sequencing studies, the jejunal and ileal luminal microbiota comprises mostly phylotypes of facultative anaerobes including streptococci, enterococci, Veillonella, lactobacilli, and Gammaproteobacteria. In the distal ileum, where transit slows, the mucosal bacterial composition changes, with an increasing proportion of anaerobic species such as Clostridiales (more specifically Clostridial clusters I, XIVa, and IV) and Bacteroidetes (124, 327, 328). It has been suggested that Streptococci and Veillonella play a key role in the ileal microbial ecosystem (43). The rapid sugar metabolism and transport systems of streptococci make them efficient competitors for substrates during transit through the small intestine (211). As a consequence, carbohydrate fermentation leads to the accumulation of lactic acid, which is further fermented to acetic and propionic acid by members of genus Veillonella, a common lactate-utilizing microbial group in the human intestine (84, 270).

**Microbiota of the lower intestine**

The human lower intestine, which consists of the colon and cecum, is the most densely inhabited microbial ecosystem known, with approximately $10^{11}$ to $10^{12}$ microorganisms per ml of intestinal contents (161, 266). Due to the low redox potential prevailing in this environment, the numbers of obligate anaerobic microbes are several orders of magnitude higher than those of aerobes or facultative anaerobes (197). Dietary compounds and intestinal secretions that are not absorbed in the small intestine to some extent reach the large bowel, where they provide the dense microbial population with nutrients and energy for growth (67). Hence, carbohydrate metabolism is of great importance in the large intestine, since the vast majority of microorganisms in the colon are saccharolytic.

In recent years, the application of high-throughput sequencing approaches has provided new insights into characterization of the human large intestine microbial composition (4, 97, 107, 157, 301, 315). Eckburg and co-workers (87) were the first to utilize an extensive culture-independent sequencing methodology for the analysis of microbial composition in human fecal samples and several colonic sites in three healthy individuals. The majority of sequences affiliated with the Firmicutes and Bacteroidetes phyla, and over 70% of the phylotypes discovered belonged to the strictly anaerobic clostridial group of Firmicute bacteria, mostly in Clostridial clusters XIVa or IV (60, 87). It is noteworthy that most of the bacterial sequences obtained corresponded to uncultivated species and novel microorganisms. The minor phyla detected were Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia. Surprisingly, only a single phylotype of methanogenic archaeon, Methanobrevibacter smithii, was observed among the sequences obtained. The results of this study are in agreement with those of previous small-scale studies (123, 130, 328), which showed relatively similar phylum distributions for healthy adults.

In contrast to the study by Eckburg and co-workers, in which unexpectedly few sequences were associated with the Actinobacteria taxa, this bacterial phylum was the second most abundant in all six samples analyzed in a pyrosequencing survey reported by Andersson et al. (4). The Actinobacteria
were dominated by a few phylogenotypes belonging to the genus *Bifidobacterium* and to the family *Coriobacteriaceae* (4). Furthermore, an underestimation of *Actinobacteria* in feces in relation to other phyla was confirmed in a study reported by Krogus-Kurikka *et al.* (157), which employed a combination of two methods that involve the fractionation of DNA preparations based on %G+C, followed by extensive 16S rRNA gene cloning and sequencing. As a result, a significantly larger proportion of high G+C content *Actinobacteria* clones were recovered with samples subjected to %G+C profiling and fractioning compared with the unfractioned intestinal samples (157). Hence, it is evident that the contradictory results of individual studies are at least to some extent due to technical bias derived from the molecular approach applied. This is discussed in further detail in Section 2.2.3.

Current knowledge on the intestinal microbiota community structure of the human gut is largely based on fecal samples, which are used as a surrogate for intestinal luminal contents. This is mainly due to the non-invasive nature of fecal sampling (318). Therefore, the phylogenotypes identified may not necessarily represent the real picture of microbial diversity of mucosa-associated communities (87, 164, 229, 348). In fact, fecal samples may overestimate the species diversity of the large intestine microbiota due to the fact that, in addition to indigenous gut microbes, human feces are also composed of transient bacteria ingested from the environment (e.g., food, drinks, etc.) (318).

### 2.1.3 Functions of the gut microbiota

The conventional view that all microorganisms cause disease has been replaced by the insight that most microbe-host interactions are commensal (with the microbes benefiting, while the host is unharmed), or even mutualistic (in which both partners benefit) (165). The benefits of commensal intestinal bacteria are well known: they help in digestion and synthesis of dietary compounds, regulate intestinal epithelial proliferation, inflammatory immune responses and host energy metabolism, synthesize vitamins, and fill a microbiological niche that might otherwise be colonized by potentially harmful enteric microorganisms (14, 96, 112, 223, 251, 316). In return, the intestinal microorganisms are provided with secure growth conditions and a constant stream of nutrients (266).

### Energy sources of intestinal bacteria

The diet-derived complex carbohydrates not degraded in the upper intestine (e.g., resistant starches, non-starch polysaccharides) and unabsorbed sugars and sugar alcohols are the main sources of carbon and energy for bacteria in the large intestine. Dietary protein and protein from pancreatic enzymes and gastrointestinal secretions also contribute to some extent to the supply of substrates to intestinal bacteria (67). In addition, the gut microbiota degrades and processes large quantities of substances derived from the human host, such as mucus and sloughed epithelial cells. Hence, the intestinal microbiota has a substantial catalytic potential, which leads to the formation of microbial metabolites with beneficial or adverse health effects. The amounts and types of fermentation products formed by intestinal bacteria depend on the relative amounts of each substrate available and the fermentation strategy (biochemical characteristics and catabolite regulatory mechanisms) of bacteria involved in the fermentation process (182).

### Importance of short-chain fatty acids

The principal end-products of intestinal microbial fermentation are short-chain fatty acids (SCFAs) such as acetate, butyrate, succinate, propionate, and lactate (133, 308).
Up to 95% of the SCFAs produced during carbohydrate fermentation are utilized by the host, providing 5 to 15% of human total energy requirements (32, 68, 207). Various tissues in the body are able to metabolize SCFAs for energy generation. Acetate and propionate provide energy for brain, muscle, and heart, while butyrate is the preferred energy source of the colon epithelial cells, providing 60 to 70% of their daily energy requirements (95, 106, 256).

Among the SCFAs, butyrate has gained particular interest, since it is known to regulate cellular differentiation and proliferation within the colonic mucosa and is suggested to lower the risk of colon cancer (12, 193, 248, 333). It has also been suggested that butyrate plays a role in inflammatory bowel diseases due to reduced capacity of the colonocytes to oxidize butyrate in the active phase of the disease (39). Although butyrate production is distributed across many Clostridial clusters of the phylum Firmicutes (245), it is mainly produced by members of Roseburia spp. and Eubacterium rectale, both members of the Clostridial cluster XIVa, as well as by Faecalibacterium prausnitzii-like organisms of Clostridial cluster IV (23, 131, 175, 245). These bacterial groups are susceptible to changes in dietary carbohydrate intake, which has an effect on colonic butyrate concentrations (85).

Lactic acid is the strongest of the common SCFAs produced by GI bacteria and therefore it tends to reduce residual pH more than other SCFAs. Lactate produced by lactic acid bacteria is rarely present in the lower intestine in high quantities, since it is normally rapidly absorbed from the intestine or used as a substrate for lactate-utilizing bacteria, such as representatives of the genera Eubacterium, Anaerostipes, Veillonella, and Megasphaera (29, 121, 131). However, in certain conditions, abnormal accumulation of lactic acid occurs as a result of carbohydrate malabsorption in the upper intestine and subsequent overgrowth of lactic acid-producing bacteria due to increased delivery of readily metabolized carbohydrates to the colon (84). Consequently, production of lactic acid exceeds the buffering and absorptive capacity of the colon and the pH of the habitat decreases, with concomitant inhibition of the activity of lactate utilizers. This cascade of events may eventually lead to impaired well-being of the host. In two recent studies, by Tiihonen et al. (307) and Mäkivuokko et al. (185), the lactic acid concentration and lactate-producing Lactobacillus and Enterococcus counts were both elevated in fecal samples from elderly subjects, whereas the numbers of butyrate-producing bacteria (members of Clostridial cluster XIVa) and lactate-utilizing bacterial species were lower than in young adults. In addition, lactate accumulation and subsequent erosion of the colonic mucosa has been shown to increase the risk of inflammatory bowel diseases such as ulcerative colitis (UC), in which concentrations up to 100 mmol per liter of lactate in fecal samples have been reported (136, 325).

Protein and amino acid fermentation in the colon

The large intestine has been described as a site of intense protein turnover (181). The active proteolytic microbial community in the large bowel includes species belonging to the genera Bacteroides, Propionibacterium, Clostridium, Fusobacterium, Streptococcus, and Lactobacillus (180). Once carbohydrate sources are exhausted in the proximal colon, sources of protein material are fermented and metabolized to salvage energy (103). Although proteins and amino acids provide a less significant energy source in the large intestine, their importance lies mainly in the formation of potential systemic toxins and carcinogens as a result of protein or amino
acid fermentation by putrefactive bacteria. Common examples of undesired metabolic end-products include phenols and indoles (as a result of anaerobic fermentation of the aromatic amino acids tyrosine and tryptophan by colonic bacteria), ammonia (as a result of oxidative or reductive deamination of amino acids) and amines (as a result of amino acid decarboxylation in the colon) (140). Production of these compounds is inhibited or repressed in many intestinal microorganisms by a fermentable source of carbohydrates. Hence, the putrefactive processes become quantitatively more pronounced in the distal bowel, where the availability of carbohydrates is more limiting (6).

**Amino acid degradation by sulfate-reducing bacteria**

The metabolism of sulfur in the human large intestine has gained much attention, since emerging evidence suggests that reduced sulfur compounds such as hydrogen sulfide (H₂S) are toxic to colonic epithelial cells and inhibit butyrate oxidation in colonocytes (242, 255). Accordingly, it is proposed that sulfide is involved in the etiology of colon cancer and ulcerative colitis (143, 242, 255). The principal sources of sulfur are the diet-derived sulfur-containing amino acids methionine, cysteine, cystine and taurine as well as endogenous sulfated mucopolysaccharides derived from the intestinal epithelial tissue, from which sulfides are formed as a result of bacterial degradation by the specialized class of gram-negative anaerobes known as sulfate-reducing bacteria (SRB) (39, 104). The SRB combine oxidation of a wide range of substrates, including hydrogen, short-chain fatty acids, and other organic acids, alcohols, and amino acids, to reduce sulfur and sulfur-containing compounds to hydrogen sulfide (39, 267). The SRB include a variety of morphologically and nutritionally different species belonging to the genera *Desulfotomaculum*, *Desulfovibrio*, *Desulfobulbus*, *Desulfobacter*, and *Desulfovomonas*. The most predominant genus, however, is *Desulfovibrio*, which is commonly isolated from environmental sources, but is also present in the digestive tract of animals and humans (28, 104, 336).

**Functions of intestinal mucus**

A mucus layer covering the GI tract offers several ecological advantages to intestinal bacteria, as it represents a direct source of nutrients bacterial growth, especially in the colon where carbon sources are limited (72). Moreover, the mucus layer contributes to host defenses, providing a protective barrier for the underlying epithelium against pathogenic microorganisms and chemical, physical, or enzymic damage (171). Mucus is a viscous gel mainly composed of high molecular weight, heavily glycosylated proteins termed mucins, which are produced by intestinal epithelial tissues. It has been estimated that 1 to 5% of colonic microbiota is able to degrade host mucin using enzymes (e.g., glycosidases and sulfatases) that can degrade the oligosaccharide chains (53, 135). The question of which bacterial species dominate in the breakdown of mucin in the mixed gut ecosystem has remained difficult to answer. Based on their capacity to grow on mucin-containing media, certain members of the genera *Akkermansia*, *Ruminococcus*, *Bacteroides*, *Bifidobacterium*, and *Clostridium* have been shown to degrade mucin (72, 263). Moreover, in a study conducted by Leitch et al. (162), the most commonly recovered sequences from mucin in an anaerobic in vitro continuous flow system were from *Bifidobacterium bifidum* and uncultured bacteria related to *Ruminococcus lactaris*. By measuring the release of reducing sugar monomers from the mucin polymer, it was observed that only mixed cultures of fecal bacteria were able to degrade mucin by more than 90%, whereas pure cultures of
Bacteroides fragilis, Bifidobacterium longum, and Clostridium perfringens showed only partial degradation (337). It is therefore likely that in vivo, consortia of bacteria are required to achieve efficient degradation of the complex structure of mucin.

2.1.4 Role of gut microbiota in disease
The intestinal microbial community of healthy humans is remarkably stable over time within individuals, indicating that certain mechanisms promote the preservation of desirable bacteria in the gut environment (345). However, when disturbed, the microbiota has been shown to play an essential role in either the etiology or maintenance of a multitude of intestinal or other health problems. There is increasing evidence to suggest that gut and immune-related diseases, such as colorectal cancer, Crohn’s disease (CD), autism, allergy, and Type II diabetes, are associated with a disturbed and unbalanced gut microbiota. A major factor in health is the balance of bacterial numbers in the gut; if the numbers grow too high or low, this can result in impaired mucosal homeostasis or altered immune regulation (271).

Antibiotic treatment leads to imbalanced microbiota
The microbial balance in the gut is known to be altered by antibiotic treatment, which facilitates the proliferation and colonization of opportunistic pathogens in ecological niches previously unavailable to them by breaking the alliance of resident microbiota. Several studies have shown the adverse effects of different antibiotics on the human gut microbiota in subjects (25, 70, 73, 144, 145, 300). The impact of antibiotic administration often lasts for a long time after discontinuation of treatment, which is observed as a prolonged dysfunction and imbalance of the commensal gut microbiota (66, 73, 144, 172). One of the frequent consequences of antibiotic therapy is antibiotic-associated diarrhea (AAD), which is commonly a result of Clostridium difficile overgrowth in the human colon (11, 204). It is estimated that C. difficile may be the inciting bacterial agent in 25 to 50% of cases of AAD, and it has become the most common nosocomial diarrheal pathogen in hospital patients (51). Pseudomembranous colitis caused by C. difficile in the mucosa of the large intestine can result in a severe inflammatory response and the destruction of the mucosal lining (257).

Aberrant gut microbiota is a risk factor in allergy
It has been suggested that perturbations of the gastrointestinal microbiota in early life delay proper maturation of the immune system, increasing the occurrence of allergic hypersensitivity (224). The gut microbiota stimulates the immune system and ‘trains’ it to respond properly to antigens. Thus, an immature microbiota, a consequence of changes in diet or antibiotic use, leads to an inadequately trained immune system, which overreacts to antigens (38). Epidemiological surveys have revealed that the intestinal microbial composition of infants and young children who have, or later develop, allergies is different from that of non-allergic children. In a study by Björkstén et al., reduced levels of Bifidobacterium and Enterococcus spp. were shown to be correlated with allergic symptoms in the first month of life, but by 6 months of age, Enterococcus spp. levels returned to those observed in healthy infants (38). Conversely, a higher Bacteroidetes to Bifidobacterium spp. ratio was reported at 2 years of age in infants having atopic symptoms (295). At the species level, Bifidobacterium adolescentis was found to be protective against atopic disease, whereas Bifidobacterium longum was shown to be an
increased risk in a study reported by Sjögren et al. (278).

**Gut microbiota linked to obesity**

Obesity is a growing problem in the developed world, resulting in numerous health complications. It has recently been demonstrated that the intestinal microbiota is involved in the regulation of energy homeostasis of the host, and thus in promoting obesity. For example, in a study by Bäckhed et al. (16), germ-free mice were shown to be resistant to diet-induced obesity. Several metabolic mechanisms underlying this resistance, including decreased absorption of glucose and generation of SCFAs from the gut lumen, increased fatty acid oxidation, and decreased deposition of triglycerides in adipocytes, have been reported (14, 317, 324).

Furthermore, obesity has been suggested to be associated with an altered gut microbial composition in both mice and humans. A study with genetically obese (ob/ob) mice showed a 50% reduction in the abundance of Bacteroidetes and a corresponding increase in the proportion of Firmicutes compared with lean, wild-type animals (167). In agreement with this, the proportion of Bacteroidetes 16S rRNA gene sequences, detected from PCR amplified clone libraries, was also reduced in fecal samples of obese human subjects, but this proportion apparently increased gradually over a 52-week weight loss period (169). Turnbaugh and co-workers (315) discovered lower levels of Bacteroidetes and more Actinobacteria phylotypes in obese individuals compared with lean, but no differences for Firmicutes, contrary to previous observations. As the vast majority of the phylotypes present in the gut microbiota belong to the Firmicutes and the Bacteroidetes divisions, the dramatic shift in microbiota composition observed in obese individuals indicates that substantial changes are being made to the functional gut ecosystem. Indeed, characterization of the gut microbiome of ob/ob mice and their lean counterparts has revealed that the obesity-associated microbiota harbors an increased number of genes encoding enzymes involved in the digestion of dietary polysaccharides (317).

The abovementioned findings originate from experimental studies carried out using rather small and defined groups of volunteers or model animals. However, in a study by Schwertz et al. (269), a total of 98 subjects (lean, overweight, and obese) were recruited for investigation of the intestinal microbiota. The results showed that the total amount of SCFAs was significantly higher in the obese subject group than in the lean subject group. The proportion of individual SCFAs changed in favor of propionate in overweight and obese subjects. Surprisingly, the ratio of Firmicutes to Bacteroidetes changed in favor of the Bacteroidetes in overweight ($p<0.001$) and obese subjects ($p<0.005$). In addition, Duncan et al. (83) detected no difference between obese and non-obese individuals in the proportion of Bacteroidetes analyzed in fecal samples, and no significant change in the percentage of Bacteroidetes in feces from obese subjects on weight loss diets. Therefore, these results contradict previous reports regarding the contribution of these two dominant bacterial phyla to the development of obesity.

**Gut microbiota play an important role in IBD**

Inflammatory bowel disease (IBD), which includes Crohn’s disease (CD) and ulcerative colitis (UC), has long been suspected to involve an abnormal reduction in immune tolerance and subsequent overreaction of the host’s immune system to gut microbiota. The diseases differ in development, prognosis and treatment, but are characterized by chronic or episodic inflammation of the gastrointestinal
mucosa. Several factors may contribute to the abnormal reactivity of the mucosal immune system against enteric bacteria, including genetic susceptibility, loss of barrier integrity of the intestinal mucosa, and a microbial imbalance in the gut ecosystem (111, 112, 265). While single factors responsible for IBD have not been discovered, numerous studies have shown an alteration in the composition of the gut microbial community in IBD, including both CD and UC (75, 97, 164, 190, 272, 281, 334). The majority of these studies have reported a more pronounced imbalance in the microbiota compared with the healthy controls. This, dysbiosis is thought to increase the vulnerability of the gut mucosa and is possibly a factor in the development of IBD. Moreover, several recent studies have noted reductions in the numbers of butyrate-producing *Faecalibacterium prausnitzii* in both CD and UC patients (97, 198, 280, 296). Hence, it has been suggested that *F. prausnitzii* may be an important bacterial species not only for its provision of butyrate to the host, but also for its anti-inflammatory effects contributing to colonic health (282). However, while an aberrant gut microbiota has been shown to be associated with IBD, it is still unclear whether alterations are the cause or the consequence of the disease.

Several other health concerns such as irritable bowel syndrome (IBS) (261), colorectal cancer (205), autism (94), and Type 1 diabetes (320) have been linked to the microbial residents of the digestive tract. The contribution of intestinal microbiota to the development of IBS is discussed in detail in Section 2.3.5.

### 2.1.4 Role of intestinal microbiota against enteric pathogens

The mucosal surface of the adult human GI tract extends up to 300 m² and is the largest body surface in contact with the external environment. It is a complex ecosystem combining the GI epithelium, immune cells and resident microbiota (203). The host is normally protected by the physical barrier created by colonized commensal microbes, which protects the epithelium from attack by invading harmful microorganisms or opportunistic species usually present in the gut in low numbers. However, the intestinal mucosa can be exposed to various enteric pathogens. In the first step of the infection process, pathogenic microorganisms adhere to the brush border of intestinal cells, enabling them to exploit the underlying signaling pathways (45, 310). Furthermore, some enteric microbial pathogens have developed specialized systems to produce virulence factors. After normal host-cell processes have been weakened, these systems enable the pathogen to penetrate and cross the epithelial barrier (65). The enteric microbial pathogens commonly target the host cell cytoskeleton during the cell penetration step. It is exploited for various purposes such as gaining entry into cells, moving within and between cells, and forming vacuoles in order to create a specialized niche, which facilitates the pathogen's chances of survival and ability to proliferate (110).

The role of the commensal GI tract microbiota in suppressing pathogen colonization is likely to be multifactorial. In addition to the physical colonization resistance, the microbiota produces a wide variety SCFAs, which are bacteriostatic for a subset of bacterial species either directly or by reducing pH of the intestinal environment. Some members of the microbiota also generate bacteriocins, small peptide molecules with microbicidal or microbistatic properties (64). In addition, intestinal microbes apply a delicate microbe-microbe signaling network against pathogenic invaders, as well as using it to optimize the composition and numbers of appropriate members of the microbial ecosystem. This coordinated
bacterial communication, termed quorum sensing, can occur within a single bacterial species or between diverse species, and enables the regulation of different processes, especially when environmental conditions are appropriate (149). A variety of different molecules can be used as signals. Common classes of soluble signaling molecules include oligopeptides in Gram-positive bacteria, N-acyl homoserine lactones in Gram-negative bacteria and a family of autoinducers known as autoinducer-2 in both Gram-negative and Gram-positive bacteria (213).

2.2 CULTURE-INDEPENDENT TECHNIQUES FOR MICROBIOTA CHARACTERIZATION

In the past, intestinal microbiota was studied using conventional culturing techniques, which apply differential media to select for specific populations of bacteria based on their metabolic requirements. Although the culture-based techniques enable the recovery of microbial isolates for further biochemical and physiological investigations under both anaerobic and aerobic conditions, only a minority (10-50%) of the bacterial species present in the human intestine are estimated to be cultivable under standard laboratory conditions (3, 87, 323). Moreover, the samples to be examined require immediate processing and the results are greatly affected by media and growth conditions. These evident drawbacks of culture-based techniques have triggered a search for novel culture-independent nucleic acid-based strategies to give a less biased view of the total microbial diversity in the gastrointestinal tract.

2.2.1 Characteristics of 16S rRNA gene

The molecular techniques initially applied in the characterization of complex marine and soil communities have revolutionized our understanding of the gut microbiota ecosystem (87, 101, 130, 292). These modern methods mainly use the ribosomal RNA (rRNA) encoding gene as a marker of genetic diversity. The ribosome functions in the translation of RNA into protein, by assembling amino acids into polypeptide chains. It consists of two subunits, both of which are clusters of proteins and structural rRNA molecules. In prokaryotes, the gene encoding the rRNA of the small ribosomal subunit has properties that make it extremely suitable for phylogenetic analysis (226, 341). Due to its pivotal role in protein synthesis ribosomes, 16S rRNA gene is ubiquitous in all prokaryotic organisms. Moreover, the gene contains several conserved regions (Figure 1), which have remained constant throughout evolution in nearly all bacterial species. Therefore, the universality of these gene fragments makes 16S rRNA gene an ideal target in applications where the intention is to analyze the total bacterial community structure in different environments. In addition to conserved regions, the 16S rRNA gene also harbors

![Figure 1. Organization of hypervariable and conserved regions within the 16S rRNA gene.](image-url)
hypervariable regions (Figure 1), which contain specific sites unique to individual bacteria (341). This uniqueness enables taxonomic positioning as well as identification of bacteria, in most cases to the species level. Hence, by measuring the occurrence or relative abundance of variants of this gene, a community profile can be obtained and compared with profiles from other intestinal tract samples (292). During the past decade, 16S rRNA gene-based techniques have allowed identification of a large number of previously unseen unculturable bacterial phylotypes and have rapidly enhanced our knowledge of phylogenetic relationships between microbial taxa (139, 252). As a result of intensive research and the introduction of high-throughput technologies, publicly available databases have shown a dramatic increase in ribosomal DNA sequences. Over 1.5 million 16S rRNA gene sequences have been deposited to date (February 2010), which is far more than for any other gene.

A number of DNA-based techniques have been applied to analyze the composition, abundance and function of the GI microbiota. The preferred analytical technique depends entirely on the purpose of the study, as well as the time and cost restrictions associated with the task (271). A variety of molecular techniques (summarized in Table 1) are available to study gut microbial communities. The respective advantages and limitations of the methods applied in the research studies of our department are discussed below in more detail.

2.2.2 Sequencing analysis of 16S ribosomal DNA populations

Full-length Sanger sequencing
Cloning and sequencing of the full-length 16S rRNA genes in a population sample provides sequence-level information on the predominant bacterial species present in the gastrointestinal tract. The Sanger sequencing technique applies dideoxy analogues of deoxynucleoside triphosphates, which terminate polymerization at a known base (264). To determine the microbial diversity in a mixed community, the PCR products amplified with oligonucleotide primers targeted to the conserved regions of the 16S rRNA gene need to be analyzed by constructing a clone library and sequencing an appropriate number of individual clones (226). The microbes present in a community specimen can be identified by comparison of their 16S rRNA gene sequences against previously analyzed sequences listed in public databases. Depending on the similarity of the newly isolated sequences, microorganisms can be more or less precisely identified. By convention, 16S rRNA gene sequences with ≥99% identity (%ID) are considered to be from the same ‘species’, while different species of the same genus have ≥97 %ID (253).

Sanger sequencing for full-length 16S rRNA genes was first applied in the exploration of human intestinal microbiota by Suau and co-workers (292), who carried out a small-scale sequencing study of 284 16S rRNA gene clones derived from one fecal sample and classified the clones into 82 molecular species. The most comprehensive full-length 16S rRNA gene cloning and sequencing study to date is a work performed by Eckburg et al. (87), in which more than 13,000 sequences of 16S rRNA genes were determined in intestinal samples of three healthy subjects.

A fundamental drawback of broad-range ‘universal’ PCR-dependent cloning and sequencing methods is the low resolution, i.e., they are only sensitive enough to detect the most abundant taxonomic groups. Moreover, the construction of clone libraries is prone to bias, which may further contort the library structure (42).
Table 1. Examples of DNA-based techniques used for intestinal microbiota analysis.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Brief description</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGGE/TTGE</td>
<td>Gel separation of PCR amplicon according to their G-C content</td>
<td>Bands can be excised for further analysis; a good basis to compare communities</td>
<td>PCR bias; low resolution; no phylogenetic identification</td>
</tr>
<tr>
<td>T-RFLP</td>
<td>Fingerprinting of microbial communities based on the digestion of terminally labeled amplicons</td>
<td>A good basis to compare communities; relative quantification; fast</td>
<td>PCR bias; limited resolution; no phylogenetic identification</td>
</tr>
<tr>
<td>FISH</td>
<td>Detection of DNA in cells by using probes that are tagged with a fluorescent dye</td>
<td>No PCR bias; assays can be designed to target bacterial species of groups</td>
<td>Low resolution; no community-wide surveys; a priori sequence information required</td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>Quantification of sequence-specific PCR products in real-time during the amplification process</td>
<td>Highly sensitive; assays can be designed to target bacterial groups and species</td>
<td>a priori sequence information required; not perfect for community-wide surveys</td>
</tr>
<tr>
<td>DNA microarrays</td>
<td>Hybridization of amplicons against 16S rRNA gene probes on a chip</td>
<td>Fast screening approach in clinical approaches; semiquantitative</td>
<td>PCR bias; detection limited to the sequences on a chip; cross-hybridization issues</td>
</tr>
<tr>
<td>Sanger sequencing of cloned amplicons</td>
<td>Cloning of 16S rRNA gene amplicons followed by capillary sequencing</td>
<td>Read length &gt; 1000 bp; moderate resolution; phylogenetic identification</td>
<td>PCR and cloning bias; laborious; not high-throughput</td>
</tr>
<tr>
<td>Direct sequencing of 16S rRNA gene amplicons</td>
<td>Massively parallel sequencing of 16S rRNA gene amplicons immobilized on beads or a slide</td>
<td>High-throughput; no cloning bias; quantitative; high resolution</td>
<td>PCR bias; maximum read length &lt; 500 bp; computationally intense</td>
</tr>
<tr>
<td>%G+C profiling</td>
<td>Microbial profiling based on the DNA migration in density gradient centrifugation according to %G+C</td>
<td>No PCR bias; no sequence information needed; fractions can be further analyzed</td>
<td>No phylogenetic identification; laborious; requires large amounts of DNA</td>
</tr>
<tr>
<td>Metagenomics with high-throughput sequencing</td>
<td>Shotgun sequencing of complete genomes present in microbial ecosystems</td>
<td>No PCR bias; high resolution; provides information of the functional genes</td>
<td>Dependent on genome assembly and annotation; computationally intense</td>
</tr>
</tbody>
</table>
Pyrosequencing
Sequencing approaches based on cloning are relatively tedious and thus not optimal for microbial community analysis of large numbers of samples. In contrast, significantly higher throughput with lower cost is offered by massively-parallel cloning-free pyrosequencing of shorter fragments of the 16S rRNA gene amplicons. Pyrosequencing generates large numbers of 16S rRNA gene sequence tags by amplifying selected variable regions within the 16S rRNA gene. It is capable of sequencing 25 million bases in one 4-hour run, achieving significantly higher throughput than Sanger sequencing (192). The shorter sequence reads require only targeted amplification of select highly variable regions of the 16S rRNA gene, which means that low-abundance bacteria can also be detected (119). Due to its massive throughput capacity, pyrosequencing enables the sequencing of multiple variable regions in the same run, which further facilitates taxonomic identification of the sequences obtained (294). In addition, the latest updates of pyrosequencing techniques enable the utilization of so-called barcoded primers, which allow parallel analysis of multiple samples in a single sequencing run. Barcoding allows specific sequences to be traced back to the original samples from which they were derived (36, 234).

During the past five years, pyrosequencing has been widely applied in human intestinal bacterial community studies. Recent examples include comparisons of gut communities between obese and lean twin pairs (315) or twin pairs concordant or discordant for CD or UC (334), as well as evaluation of microbiota alterations and stability during and following the use of antibiotics (73, 144). Moreover, Andersson et al. (4) demonstrated the applicability of pyrosequencing for the assessment of microbial communities in different compartments of the GI tract while Wu et al. (343) analyzed fecal samples from 10 individuals and compared methods of storage, DNA purification and sequence acquisition.

Although pyrosequencing has proved to be a valuable tool in intestinal microbial ecology studies of the human GI tract, it does have certain shortcomings. The most apparent limitation is the short length of sequence reads (<500 bp versus 1 500 bp in Sanger), which limits full length bacterial 16S rRNA sequence assembly. In addition, the technique is more error-prone (5-10 errors/kb) than conventional Sanger sequencing (0.01 errors/kb). The errors in pyrosequencing reads typically take the form of one-base indels along with substitutions and ambiguous bases, and occur most often in homopolymeric regions of the sequences (88).

2.2.3 Percent guanine + cytosine profiling
Percent guanine + cytosine (%G+C) profiling is a method for initial investigation of bacterial populations of previously unknown structure (129). It has been shown to be powerful for studying whether different factors such as environmental conditions (129, 225), diseases or disorders (75, 150) or diet composition (7, 8, 186) have any effects on microbiota composition. The approach is a profiling method, which does not give exact bacterial names. However, it provides taxonomically relevant characteristics of the component bacteria, which facilitates the subsequent more detailed analyses. Percent G+C profiling is based on two essential points. Firstly, each bacterial species has a characteristic G-C content in its chromosomal DNA, which is known for all bacterial species described. Secondly, the proportion of guanine + cytosine in chromosomal DNA affects its density, G-C-rich DNA being denser than A-T-rich DNA. Based on this characteristic, DNA
with high %G+C migrates in density gradient centrifugation differently from that with low %G+C. Finally, DNA with different G-C content is quantified by UV absorbance. The great benefit of this method compared with all other DNA-based methods is its total lack of dependence on any a priori information on the bacteria being analyzed. The profile obtained is completely unbiased, because no oligonucleotide primers or probes essential for the majority of DNA-based analytical methods are required.

In addition, the method can be used as a pre-processing treatment to fractionate the pool of bacterial chromosomal DNA with any G-C content for the subsequent detailed analyses including 16S rRNA gene sequencing of microbial community samples (128, 150, 157, 225). Fractionating the total bacterial DNA preparations according to the %G+C content minimizes the bias typically involved in multi-template PCR reactions of complex bacterial communities and hence allows less abundant species to be amplified by bacterial 16S rRNA gene-targeted broad-range PCR prior to sequencing. This is especially significant in the amplification of sequences with high DNA G-C contents (e.g., Actinobacteria %G+C ≥ 60%) from microbial communities, when low G-C content sequences with considerably higher efficiency of template dissociation are not competing in the same reaction (339). In a study by Krogis-Kurikka et al. (157), the initial %G+C profiling step prior to cloning and sequencing was shown to significantly increase the sequence diversity obtained for bacteria with high chromosomal G-C content from human fecal bacterial DNA specimens.

2.2.4 Quantitative real-time PCR

Pitfalls of end-point PCR in quantification
Conventional polymerase chain reaction (PCR) uses agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction (218). Theoretically, there is a quantitative relationship between the amount of starting target sequence and the amount of PCR product for any given cycle, but in real life the amplification is exponential only up to a certain point. During the reaction some necessary components usually become limiting (e.g., amount of primers, polymerase activity, re-annealing of the products). The precise quantification of differences in the amount of starting material in end-point PCR suffers from the plateau effect during the later stages of the PCR reaction (Figure 2). At this point the reaction has stopped and no more products are made. Due to the plateau effect, end-point PCR is not a convenient method for quantitative analyses in which the initial amount of target template is of interest.

Real-time PCR procedure
Quantitative real-time PCR is a powerful advancement of conventional end-point PCR, enabling quantification of the target nucleic acid (126). Generally, the technique has exceptional performance characteristics, including a wide dynamic range (up to seven orders of magnitude) and high sensitivity. Therefore, it is a superior method for the accurate and targeted quantification of specific bacterial species or groups within microbial populations. The major aspect differentiating real-time PCR from conventional PCR methodologies is that target copy number is determined from the fractional cycle at which a threshold amount of amplicon DNA is reached, but is still within the exponential phase of the amplification (217). This approach ensures that interfering factors associated with the late stages of amplification are minimized and provides the potential for unprecedented precision for quantitative determinations.

The real-time PCR system is based on the detection and quantification of a
fluorescent reporter. This signal increases in direct proportion to the amount of amplified PCR product in the reaction. The higher the starting copy number of the nucleic acid target, the sooner an increase in fluorescence signal exceeding the threshold value is observed (Figure 2). Hence, the detection system allows the exponential phase to be monitored in order to calculate the amount of target-template present in the sample at the start (340).

**Absolute quantification with a standard curve**

In absolute quantification, real-time PCR reaction is performed on both the experimental samples and reference standards to determine the concentration of a target gene present in a particular sample. To construct a standard curve, a template with known concentration is required, which is subsequently diluted to create a suitable range of standard concentrations. The calibration curves can be based on DNA standard molecules such as recombinant plasmid DNA, genomic DNA, or PCR product (260). The unknown test samples are amplified in parallel with the standards in the same experimental run. Based on the amplification of the standards, absolute numbers of the target organism in the sample can be interpolated (Figure 3).

Typically, the standard curve is illustrated as a plot of the threshold cycle ($C_T$) values of standard dilutions against the logarithm of the amount of bacterial DNA added. Linear regression analysis is used to determine the slope and intercept, which correspond to the amplification efficiency and the number of amplicon molecules at threshold, respectively.

Standard curves allow the generation of highly specific, sensitive, and reproducible data. However, the external calibration curve model has to be carefully validated, as quantitative accuracy depends entirely on both the accuracy of DNA standard quantification and the quality of standard curve construction (98).

---

**Figure 2. Amplification plot showing exponential and plateau phases.** The vertical axis represents fluorescent signal and the horizontal axis PCR cycle number. Fluorescent threshold (green line) provides the reference point from which the threshold cycle ($C_T$) is calculated. The lower a $C_T$ value, the more copies are present in the specific sample. When plotted on a linear scale, as above, the curve has a sigmoidal course with an exponential phase and a plateau phase.
Fluorogenic detection strategies

The most common detection strategies in real-time PCR are i) SYBR Green I, ii) TaqMan probes, and iii) Molecular beacons. All of these chemistries allow detection of amplified PCR products via the generation of a fluorescent signal.

i) SYBR Green I

SYBR Green I is the simplest and most economical real-time PCR detection chemistry. It is an asymmetrical intercalating cyanine fluorescent dye specific for double-stranded (ds) DNA, which binds to the minor groove of the DNA double helix. In solution, the unbound dye exhibits very little fluorescence, but the fluorescence is greatly enhanced upon DNA-binding. The resulting DNA-dye complex absorbs blue light (λ<sub>max</sub> = 488 nm) and emits green light (λ<sub>max</sub> = 522 nm). During real-time PCR reaction, SYBR Green binds to the amplified dsDNA and emits a fluorescent signal (Figure 4). As a PCR product accumulates in each cycle, the fluorescence signal increases. Detection of this signal enables the direct quantification of amplified

Figure 4. Principle of SYBR Green I chemistry. At the beginning of PCR cycle the DNA is denatured and the dye is unbound. During elongation the dye molecules bind to the newly synthesized double-stranded DNA, resulting in a dramatic increase in light emission by SYBR Green I molecules upon excitation.
DNA without any labeled probes. Due to its flexibility in that no additional oligonucleotide probes are required, SYBR Green I has great potential in applications where a diverse target population is to be quantified with PCR (13). Note, however, that SYBR Green I is non-specific, i.e., the dye cannot distinguish between specific and non-specific products (e.g., primer-dimers and misprimed products) accumulated during PCR reaction. Therefore, careful primer design and assay optimization are critical factors in SYBR Green I-based approaches.

The use of SYBR Green I chemistry enables a melting curve analysis to determine the melting temperature ($T_m$) of the newly synthesized PCR products. Every piece of double-stranded PCR product has a melting point at which 50% of the DNA is in single-stranded form. The melting point temperature depends on the length, sequence order, and G-C content of the amplicon. Basically, organisms with higher G-C content dissociate and form a melting peak at higher temperature compared with the PCR product of organisms or artefacts with lower G-C content. The principle is similar but not equivalent to agarose gel electrophoresis, where the separation is based primarily on amplicon length. Melting curve analysis is normally carried out in conjunction with each real-time PCR assay to distinguish the fluorescent signal obtained from the specific amplification product from mispriming artefacts such as primer-dimers.

**ii) TaqMan probes**

TaqMan probes are dual-labeled oligonucleotides that contain a covalently attached fluorescent dye located on the 5’ base and a quenching dye located on the 3’ base. Hence, the fluorescent signal detected derives solely from the amplification of the probe-incorporated PCR product, which increases the assay sensitivity compared with SYBR Green I-based approaches in which non-specific products are also detected. This is essential especially in reactions starting with small amounts of template DNA (125, 187). On the other hand, since probe-based methodology requires a binding site for the probe in close proximity to one of the primers, such a conserved site is likely to be missing when the intention is to amplify larger taxonomic groups in a single real-time PCR assay.

During PCR the probe hybridizes to an internal region of the amplicon. When the dye and quencher are in close proximity, the fluorescent dye transfers energy to the

![Image](image.png)

**Figure 5. Principle of fluorescence resonance energy transfer (FRET).** As long as the TaqMan probe is intact and the reporter and the quencher dyes are in close proximity, no fluorescence signal is emitted due to the quenching effect.
quenching dye molecule, resulting in no detectable fluorescence (Figure 5). This is known as fluorescence resonance energy transfer (FRET) (57).

Subsequently, during the extension phase of PCR amplification, the 5’-3’ exonuclease activity of the DNA polymerase degrades the 5’ end of the probe. The cleavage releases the probe and separates the reporter and its quencher, resulting in an increase in fluorescence activity by the reporter dye. This allows the fluorescence of the dye to be detected (Figure 6). Fluorescence increases in each PCR cycle in proportion to the rate of cleavage, and hence to the amount of DNA template present in the reaction.

In addition to being specific detection chemistry, TaqMan probes allow multiplexing. The probes can be labeled with different, distinguishable reporter dyes, which allow the amplification of multiple distinct sequences in one reaction tube. This results in reduced reagent costs and sample use, and increased throughput. Developing multiplex real-time PCR assays can be difficult and time-consuming. As the reaction complexity increases, significant optimization may be required to generate reliable data. The most challenging task is to develop multiplex assays that amplify all targets with equal efficiency.

**iii) Molecular beacons**
Molecular beacons are similar to TaqMan probes but are not designed to be cleaved by the 5’-nuclease activity of Taq polymerase (319). These probes are tagged with a fluorescent dye on the 5’ end and a quencher dye on the 3’ end of the oligonucleotide probe. A region at each end of the molecular beacon probe is designed to be self-complementary. Hence, at low temperatures the ends anneal, creating a hairpin structure. This annealing property positions the two dyes in close proximity, quenching the fluorescence from the reporter dye (Figure 7).

The central region of the probe is designed to be complementary to a target sequence of the PCR amplification product. Therefore, at high temperatures the beacon unfolds in the presence of the complementary target sequence and the reporter dye is no longer quenched. As the temperature of the PCR is lowered, the probe sequence in the loop anneals to its target and a conformational reorganization occurs that separates the quencher from the reporter, leading to detectable fluorescence (Figure 8). This cascade of events occurs on each cycle of PCR amplification and the resulting signal is proportional to the amount of template in the reaction.

![Diagram](image.png)

**Figure 6. Fluorescence detection of TaqMan probe.** The probe is cleaved by the 5’-nuclease activity of Taq polymerase during the extension step of the amplicon. Away from the quenching dye, the light emitted from the reporter dye in an excited state is detected.
Molecular beacons are extremely specific. They enable the discrimination of target sequences that differ from one another by a single nucleotide substitution. The reason for the specificity is that they can exist in two different stable physical states. In one state, the molecular beacons are hybridized to their targets and energy is stored in the probe-target helix. In the second state, they are free in solution and energy is stored in their stem helix. Due to their specificity, molecular beacons are ideal probes for use in clinical diagnostics.

**PCR inhibitors hamper the analysis of intestinal samples**

Although the standard curve approach provides a good estimate of the efficiency of the PCR assay, it does not supply any information about the effect of the matrix of the real test sample. Biological samples are complex and may contain inhibitors...
that are not present in standards based on purified template, and this may reduce the PCR efficiency (159). Fecal matter contains several inhibitory factors such as bile salts, hemoglobin degradation products, and complex polysaccharides that can have significant adverse effects on the efficiency and sensitivity of PCR-based assays (54, 214). Fecal PCR inhibitors generally exert their effects by interacting with target DNA or blocking the enzyme activity of thermostable DNA polymerases. Hence, the best way to avoid PCR inhibition is to prevent the inhibitor from being processed with the sample by extensive purification. However, if there are residues of inhibitors remaining in the sample after purification and DNA extraction steps, the sample may in some cases be diluted to reduce the amount of inhibitory factors. In general, it is essential to estimate the PCR efficiency of the serially diluted test sample and compare the results obtained against the amplification efficiency of the standard curve (289). To obtain reliable real-time PCR results, these two efficiencies should be identical.

**Real-time PCR primer design**

Optimal design of the PCR primers is essential for accurate and specific quantification using real-time PCR. Hence, in order to obtain reliable quantitative results, the target primer sequences must be complementary only to the desired target sequence. The first step in primer design protocol is to determine the sequences that need to be targeted by the PCR. This is followed by the identification of related genes and specific regions of the templates of interest by multiple sequence alignment, after which the specificity of the individual primer candidates is verified by a DNA database search. The specificity of the 3’ terminal end in PCR primers to the desired target template is essential to eliminate non-specific amplification. This is due to the fact that Taq polymerase extension can be greatly retarded by terminal 3’ end mismatches (160). On the other hand, if primers have too stable 3’ end (clamps of 3 or more Cs or Gs at the 3’ end), they may also bind to complementary sites of the non-target template, leading to false-positive amplification (274).

Complementarity between the primer sequences applied at the 3’ end promotes the formation of primer-dimer artefacts. The creation and subsequent amplification of these template-independent artefacts reduces the availability of primers to the template molecule, resulting in decreased sensitivity or even failure of the PCR (138). The formation of primer-dimers can be reduced by careful primer design, the application of stringent PCR conditions and the use of ‘hot-start’ Taq polymerase (55, 69, 187).

Length of a primer is a critical parameter in assay development, as too short primers reduce the specificity of PCR reaction (342). The rule-of-thumb is to use primers with a minimum length of 16 bases to avoid cross-reactions with unwanted DNA sequences. On the other hand, too long primers result in slower rate of hybridization to the target DNA sequence, which directly affects the PCR efficiency. In practice, primers longer than 30 bases are rarely used. Another essential factor is the annealing temperature (T_a) of primers to the template. Too low T_a hampers the assay specificity and may lead to formation of non-specific amplification products. In general, the optimal T_a should be low enough to enable the hybridization between primer and target template, but high enough to prevent the mispriming of non-target DNA.

An important factor to consider when designing a PCR assay is the potential presence of secondary structures. If primers are designed on secondary structures of the template, which are stable even above the annealing temperatures, the primers are unable to bind to their complementary target region. This leads to decreased amplification
efficiency and the yield of PCR product is significantly affected. The likelihood of secondary structures is greatest within regions rich in complementary base pairing, such as the stem of the stem-loop structure in the template (277). Hence, it is important to design primers in the regions of the templates that do not form stable secondary structures during the PCR reaction.

Assay optimization
Although theoretical specificity and functionality of the primers is a precondition for a successful assay, it is always necessary to carry out in vitro optimization and validation. For real-time PCR, a few key components should be optimized in order to achieve feasible and reproducible results. These factors include magnesium concentration, which allows the polymerase enzyme to function at an optimal level, and primer concentrations, which affect the sensitivity and specificity of the assay (41, 156). In addition to optimization of reagents, it is essential that the specificity of any newly designed assay is tested against purified DNA from bacterial strains belonging to the target group (positive controls) and against strains outside the target group (negative controls). It is important to pay attention to the choice of these positive and negative controls in order to ensure adequate coverage of the assay in the environment of interest. The discriminatory power against negative controls depends somewhat on the case in hand, but at least a thousand-fold difference in the signal from positive and negative controls should always be maintained. As part of the validation process, annealing temperature and other crucial assay characteristics are optimized for each individual real-time PCR primer pair to enhance assay specificity and sensitivity.

Application of real-time PCR in intestinal microbiology
Both SYBR Green I and TaqMan-based 16S rRNA gene-targeted real-time PCR approaches have been applied in investigation of the human gut microbiota. The areas of application include monitoring the abundance of specific microbial groups within different compartments of the GI tract in health and disease, as well as probiotic and prebiotic research. Moreover, the technique has been shown to be a valuable tool in investigating the temporal stability of specific intestinal microbial groups, and in revealing age-related or antibiotic-associated alterations in the gut microbial community structure. Examples of studies in which 16S rRNA gene-targeted real-time PCR has been applied for intestinal microbial monitoring are listed in Table 2.

2.3 IRRITABLE BOWEL SYNDROME

2.3.1 Definition and description of the disease
Irritable Bowel Syndrome (IBS), formerly known as ‘spastic colon’, is a functional bowel disorder that occurs in the absence of identifiable organic disease or structural or biochemical abnormality. It is considered the major cause of abdominal discomfort and gut dysfunction worldwide, with a prevalence of 10-20% of the adult population, which makes it the most frequent diagnosis in gastroenterology (174, 259). The prevalence of IBS is highest in people aged between 20 and 40 years, and usually decreases with age (31, 298). Abdominal pain or discomfort is the most frequently reported symptom in IBS (116). The pain is usually relieved by defecation, which supports the colonic origin of the symptom. It is also associated with bowel dysfunction, such as chronic
Table 2. Examples of gut microbiota studies with 16S rRNA gene-targeted real-time PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Title</th>
<th>Year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENEAL OR AGE-RELATED GI PROFILING</td>
<td>Use of 16S rRNA gene-targeted for real-time PCR of predominant bacteria in human feces</td>
<td>2004</td>
<td>(199)</td>
</tr>
<tr>
<td></td>
<td>Quantification of intestinal bacterial populations by real-time PCR with a universal primer set and minor groove binder probes: a global approach to the enteric flora</td>
<td>2004</td>
<td>(230)</td>
</tr>
<tr>
<td></td>
<td>Factors influencing the composition of the intestinal microbiota in early infancy</td>
<td>2006</td>
<td>(238)</td>
</tr>
<tr>
<td></td>
<td>The <em>Firmicutes/Bacteroidetes</em> ratio of the human microbiota changes with age</td>
<td>2009</td>
<td>(194)</td>
</tr>
<tr>
<td></td>
<td>Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians</td>
<td>2010</td>
<td>(34)</td>
</tr>
<tr>
<td>IBD</td>
<td>Detection of <em>Helicobacter</em> species DNA by quantitative PCR in the gastrointestinal tract of healthy individuals and of patients with inflammatory bowel disease</td>
<td>2004</td>
<td>(141)</td>
</tr>
<tr>
<td></td>
<td>Gut-associated bacterial microbiota in paediatric patients with inflammatory bowel disease</td>
<td>2006</td>
<td>(63)</td>
</tr>
<tr>
<td></td>
<td>Estimation of faecal carriage of <em>Clostridium difficile</em> in patients with ulcerative colitis using real time polymerase chain reaction</td>
<td>2008</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>Low counts of <em>Faecalibacterium prausnitzii</em> in colitis microbiota</td>
<td>2009</td>
<td>(280)</td>
</tr>
<tr>
<td></td>
<td>Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria</td>
<td>2010</td>
<td>(243)</td>
</tr>
<tr>
<td>IBS</td>
<td>Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification</td>
<td>2009</td>
<td>(179)</td>
</tr>
<tr>
<td></td>
<td>Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers</td>
<td>2009</td>
<td>(158)</td>
</tr>
<tr>
<td></td>
<td>Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome</td>
<td>2010</td>
<td>(299)</td>
</tr>
<tr>
<td>ALLERGIES</td>
<td>Differences in developing intestinal microbiota between allergic and non-allergic infants: a pilot study in Japan</td>
<td>2007</td>
<td>(283)</td>
</tr>
<tr>
<td>OBESITY</td>
<td>Human gut microbiota in obesity and after gastric bypass</td>
<td>2009</td>
<td>(344)</td>
</tr>
<tr>
<td></td>
<td>Microbiota and SCFA in lean and overweight healthy subjects</td>
<td>2010</td>
<td>(269)</td>
</tr>
<tr>
<td>ANTIBIOTICS</td>
<td>Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota</td>
<td>2004</td>
<td>(25)</td>
</tr>
<tr>
<td>PRO &amp; PREBIOTICS</td>
<td>Microbiological effects of consuming a synbiotic containing <em>Bifidobacterium bifidum, Bifidobacterium lactis</em>, and oligofructose in elderly persons, determined by real-time polymerase chain reaction</td>
<td>2004</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td>Molecular monitoring of the fecal microbiota of healthy human subjects during administration of lactulose and <em>Saccharomyces boulardii</em></td>
<td>2006</td>
<td>(322)</td>
</tr>
</tbody>
</table>
diarrhea and/or chronic constipation, but specific symptoms may vary from person to person and time to time (79). People with IBS may also suffer from irregular bowel movements, abdominal distension, acid reflux, bloating, urgency, loss of appetite, and flatulence (77). While IBS is a chronic condition, for most people there are usually times when symptoms are worse and times when symptoms improve or even disappear (116). IBS is not considered a life-threatening disease. When diagnosed according to current criteria, IBS is associated with a good prognosis and the diagnosis is unlikely to be changed to that of an organic disease (231). However, IBS patients suffer from an excessively higher rate of co-morbidity with other disorders, such as fibromyalgia, chronic fatigue, sleep disturbances, and psychiatric disorders (24, 91, 127, 321). Hence, IBS negatively affects the quality of life of patients and causes significant direct economic costs due to the use of healthcare resource (77), and indirect costs due to work absenteeism (missed days of work) or presenteeism (loss of productivity at work). The indirect costs appear to account for most of the financial burden associated with IBS (71).

2.3.2 Diagnosis of IBS
The symptom-based diagnosis of IBS has changed over time. The first symptom criteria were presented by Manning et al. (191), who identified six symptoms that differentiate patients with IBS from those with organic intestinal diseases. A major limitation of these criteria was the difficulty in differentiating IBS from organic diseases of the lower intestine (120). However, the Manning criteria provided the basis for the more recent Rome criteria, which were initially published for IBS in 1989 by the Multinational Committee of Clinical Investigators (305). These criteria were later revised and published as the Rome I criteria. The main change from the 1989 criteria was the requirement of abdominal pain for IBS diagnosis (304). In 1998, the Rome Working Team proposed changes to the definition and diagnostic criteria for IBS to reflect new research data and to improve clarity. Accordingly, they established the Rome II criteria (306), which represented committee consensus based on research results and expert opinion. Finally, in 2006 these were further defined by the expanded Rome Working Team into the Rome III criteria, focusing on stool form over defecation frequency (174). The Rome I, II, and III criteria are presented in Table 3.

The diagnosis of IBS is based on the exclusion of organic diseases. Therefore, once the diagnostic criteria have been met, it is necessary to exclude other medical disorders, which present symptoms similar to IBS. Physicians rely on a variety of procedures and laboratory screening tests to confirm a diagnosis. The Rome II and III criteria, however, now define markers which allow professionals to diagnose IBS after a careful examination of a patient’s medical history and physical abdominal examination searching for any warning signs of organic diseases such as colonic cancer, IBD, celiac sprue, or infectious diarrhea (50, 78, 285). Warning symptoms not typical of IBS include unrelenting diarrhea, nocturnal symptoms, unintentional weight loss, symptom onset after age 50, hematochezia or a family history of organic GI disorders.

2.3.3 IBS subtypes
Using supportive symptoms, i.e., stool frequency, stool consistency, and defecatory features (straining and urgency), IBS patients can be subdivided into diarrhea-predominant (IBS-D), constipation-predominant (IBS-C), or mixed subtype (IBS-M) with alternating episodes of both diarrhea and constipation (77, 306). Each IBS subtype (IBS-D, IBS-C, IBS-M) represents about one-third of the
Table 3. Rome I, Rome II, and Rome III criteria for IBS.

Rome I criteria for IBS
At least three months continuous or recurrent symptoms of:

I. Abdominal pain or discomfort which is:
   - Relieved with defecation; and/or
   - Associated with a change in frequency of stool; and/or
   - Associated with a change in consistency of stool

And

II. Two or more of the following, on at least a quarter of occasions or days:
   - Altered stool frequency
   - Altered stool form (lumpy/hard or loose/watery)
   - Altered stool passage (straining or urgency, feeling of incomplete evacuation)
   - Passage of mucus
   - Bloating or feeling of abdominal distension

Rome II criteria for IBS
At least 12 weeks (need not be consecutive) in the preceding 12 months of abdominal discomfort or pain that has two of three features:

   - Relieved with defecation; and/or
   - Onset associated with a change in frequency of stool; and/or
   - Onset associated with a change in form (appearance) of stool

Rome III criteria for IBS*
Recurrent abdominal pain or discomfort at least three days per month in the last three months associated with two or more of the following:

   - Improvement with defecation
   - Onset associated with a change in frequency of stool

*Onset associated with a change in form (appearance) of stool

patients fulfilling the Rome II criteria (82). IBS-D is determined by predominantly loose or watery stools at least 25% of the time, whereas IBS-C is determined by predominantly hard or lumpy stools at least 25% of the time. IBS-M applies to subjects reporting alternating constipation and diarrhea for at least 25% of bowel movements (174). It is characteristic of IBS-M that after a period of constipation, frequency of bowel movements increases, leading to a period of diarrhea. These periods usually fluctuate within hours or up to week (82, 113).

2.3.4 Pathophysiological mechanisms and etiological factors in IBS
IBS symptoms cannot be traced to a single organic factor. Instead, IBS is considered a complex biopsychosocial condition in
which a multitude of mechanisms at the central and peripheral level interact (80). Visceral hypersensitivity, abnormal motor function, low-grade mucosal inflammation, food intolerance, gastrointestinal infections, psychosocial factors, alterations in gut microbiota, and genetic factors are suggested to be involved in the etiology of IBS symptoms (19). The role of intestinal microbiota in the pathophysiology of IBS is discussed in detail in Section 2.3.5.

It is evident that IBS involves hypersensitivity of the bowel that is expressed in several ways, including changes in information content carried by spinal primary afferent neurones and altered intestinal motility (48). Quantitative data from human studies have revealed that visceral hypersensitivity includes lowering the threshold distension for evoking pain in IBS patients (46). Hyper-reflexia in patients suffering from IBS has been demonstrated by a decrease in threshold distension to induce enteric reflexes and by enhanced accommodation reflexes in the colon, leading to altered gut motility (219). Pivotal psychological factors that affect the abnormal contractions of the colon are stress and anxiety (30, 241). Since the colon is controlled by the autonomic nervous system via neural pathways that connect it to the brain, in reaction to stressful events the colon often either begins violent spasmodic contractions or slows down or stops contracting. When the contractions are vigorous and protracted, any food ingested and transiting through the GI tract rapidly causes gas, bloating and diarrhea. In contrast, when contractions are weaker, the passage becomes retarded, resulting in constipation (99, 246, 273).

The role of enteric infection in the pathogenesis of IBS has been recognized for years and the development of IBS in patients who have undergone a previous episode of infectious gastroenteritis has been reported in several prospective studies (44, 206, 220, 221, 236, 254, 326). These studies indicated that 4 to 17% of patients with acute gastroenteritis develop post-infective IBS (PI-IBS) (287). Moreover, it has been estimated that the risk of developing IBS is increased after a GI infection in correlation with the severity of the enteric infection (302). Unlike sporadic non-PI-IBS, PI-IBS has a defined moment of onset. Individual studies suggest that in most patients the symptoms persist for years and gradually improve (22). Young age, prolonged fever, female gender, anxiety, and depression are considered risk factors for PI-IBS (86, 115, 221, 302). Various intestinal bacterial pathogens including Campylobacter, Escherichia coli 0157:H7, Salmonella, and Shigella have been associated with the development of PI-IBS, but it remains unclear whether all pathogens possess an equivalent risk (146, 195, 206, 208). In addition, viral gastroenteritis appears to result in a more transient form of PI-IBS than bacterial gastroenteritis (196).

Diarrhea-predominant IBS has been recently shown to be characterized by elevated colonic luminal serine protease activity. Gecse and co-workers (102) observed a significant increase in fecal serine protease activity in IBS-D patients compared with IBS-C or infectious diarrhea. In addition, the mucosal in vitro application of fecal supernatants from IBS-D patients in a mouse colon strip increased the colonic permeability, the effect of which was prevented by serine protease inhibitors and dependent on protease-activated receptor-2 (PAR-2) expression. The elevated serine protease activity in IBS-D was suggested to be derived from microbial origins (102).

It is becoming evident that low-grade inflammation of the intestinal mucosa is present in IBS patients and plays a pivotal role in gastrointestinal dysfunction. Increased numbers of mucosal lymphocytes are
associated with the development of PI-IBS and IBS-D in various lymphoid compartments of the small or large intestine in IBS patients (52, 288, 309). Moreover, there is strong evidence that systemic immune activation is involved in IBS, as elevated levels of plasma pro-inflammatory interleukin (IL)-6 and IL-8, as well as tumor necrosis factor (TNF)-α in peripheral blood mononuclear cells, have been observed in IBS patients (76, 170). O’Mahony et al. (227) discovered that the ratio of IL-10 secretion to that of a pro-inflammatory cytokine IL-12 was significantly lower in IBS patients compared with controls.

The presence of mast cells in the lamina propria of the terminal ileum and mucosa of the colon has been shown to be more pronounced in IBS patients than in healthy controls, suggesting that mast cells may affect the GI sensorimotor function and contribute to IBS symptoms (21, 228, 329). In general, extensive research has been carried out to discover robust and reliable biomarkers for IBS (56, 163, 184). However, since IBS contains a variety of conditions with different underlying causes, biomarkers and therapies need to be identified for each IBS subgroup (1).

2.3.5 Intestinal microbiota and IBS

There is increasing evidence that the gut microbiota contributes to pathogenesis of IBS. This proposition is supported by the fact that alterations have been discovered in bacterial community structure in the lower intestine of IBS patients, as discussed below. Moreover, small intestinal bacterial overgrowth (SIBO) has been reported in some patients with IBS (177, 239) and an abnormal pattern of fecal SCFAs also points to an altered microbiota (299, 312). IBS can develop following an acute GI infection, as discussed above, and therapeutic manipulation of the gut microbiota with probiotics, prebiotics or symbiotics has frequently been shown to alleviate IBS symptoms. The role of microbiota-targeting therapies in IBS is discussed in Section 2.3.6.

Early studies using conventional culture-based techniques indicated that people suffering from IBS harbor reduced numbers of Lactobacillus spp., coliforms, and Bifidobacteria spp., and have greater instability of the intestinal microbiota (18, 47). A case-control study of 25 IBS patients and 25 gender-matched controls by Si and co-workers (275) reinforced the findings on reduced bifidobacterial numbers in IBS patients, and also revealed an increase in Enterobacteriaceae compared with healthy controls. In contrast to the earlier studies, Mättö et al. (201) discovered a significantly higher number of coliforms in fecal samples of IBS patients. On the other hand, no differences in the mean cultivable numbers of bacteroides, bifidobacteria, spore-forming bacteria, lactobacilli, enterococci, or yeasts were found, but an increased aerobe to anaerobe ratio was observed (201).

Due to the obvious limitations of GI bacterial culturing techniques, during the past five years research on IBS-related microbiota screening has relied merely on the modern DNA-based analysis methods. In addition to the work presented in this thesis, one of the first studies in which molecular techniques were applied in intestinal microbiota analysis of a large group of IBS patients was published in February 2005 by Mättö and co-workers (201). They evaluated the temporal variation in the GI microbiota over a 6-month period in 21 IBS patients and 17 healthy controls using denaturing gradient gel electrophoresis (DGGE) of fecal DNA samples, complemented with 16S rRNA gene-targeted sequencing. A more pronounced temporal instability of the microbiota composition was observed among IBS patients compared with the healthy control group. However, this finding relied solely on qualitative inspection of the profiles and part of the instability was explained by
the consumption of antibiotics. Furthermore, a trend-like increase in *Clostridium* spp., as well as a reduction in *Eubacterium* spp., was detected in the IBS patients (201). The same authors also quantified the clostridial groups from the same sample set with a novel method named transcript analysis, with the aid of affinity capture (TRAC). With TRAC, IBS-C patients were discovered to harbor fewer bacteria belonging to the *Clostridium cocoides – E. rectale* (Clostridial cluster XIVa) group compared with the healthy control subjects (202). In addition, the same set of IBS samples that exhibited temporal instability in a study by Mättö et al. (201) were re-examined by excluding samples from patients recently prescribed antibiotics. As a consequence, the temporal variation in the intestinal microbiota was found to be no higher in IBS patients when analyzed with the DNA-based DGGE, but fecal RNA-derived DGGE profiles of IBS patients indicated increased instability of the bacterial population compared to the healthy control subjects (202).

A microarray-based analysis with the Human Intestinal Tract Chip (HITChip), a 16S rRNA gene-based phylogenetic microarray specifically designed to target the human intestinal microbiota, was presented in the doctoral thesis of Mirjana Rajilić-Stojanović (249) to study the fecal microbiota of primary care IBS patients. The total microbiota of IBS patients was discovered to be distinct from that of healthy controls when the respective phylogenetic profiles of the two groups were clustered. Moreover, the composition of the fecal microbiota of healthy volunteers appeared to be more homogeneous than that of IBS patients. This was suggested to be due to the multifactorial nature of IBS, as the subjects in the study included patients from all three IBS subgroups (IBS-D, IBS-C, and IBS-M). The microbial composition of IBS-D patients was observed to be the most distinct from the healthy controls. In the compositional analysis of samples representing all three IBS subtypes and the healthy control group, significant differences in 19 genus-level taxa representing *Bacteroidetes*, bacilli, and Clostridial clusters III, IV, and XIVa were discovered. The abundance of *Bacteroides* spp. was lowest in IBS-C patients, whereas IBS-D patients were characterized by higher levels of bacterial phylotypes from the order *Bacilli*. Furthermore, certain genera belonging to the Clostridial cluster IV were significantly higher in the samples of IBS-C patients compared with those of IBS-D patients. The main conclusion of the study was that the fecal microbiota of IBS patients appears to be disturbed, and also specific for each IBS subtype (249).

The first reported study applying extensive 16S rRNA gene cloning and sequencing to compare the molecular profile of the fecal microbiota of IBS patients with that of healthy control subjects was published in July 2007 (150). Initially, the bacterial DNA preparations extracted from fecal samples were pooled; 10 from IBS-D subjects, 8 from IBS-C, 6 from IBS-M, and 23 controls. The pooled DNA preparations were subsequently fractionated according to their percent G-C content and the fractions displaying the most divergence were selected for cloning and sequencing of the 16S rRNA gene. The rationale of G+C profiling was to diminish the bias favoring the most abundant organisms and to facilitate the recovery of sequences with high G+C content sequences in PCR and cloning, as well as to select the most divergent G+C fractions between pooled IBS subtypes and controls for further analysis. Accordingly, the three most variable %G+C fractions between the groups, representing bacterial genomes with %G+C 25-30, 40-45, and 55-60, were subjected to cloning and sequencing.

The results showed that in addition to differences detected in the %G+C profiling between controls and IBS subtypes, there
were significant differences between the clone libraries of pooled samples in several bacterial genera. These results were verified and complemented for the individual samples by real-time PCR with assays designed to target the most differing phylotypes between the libraries. As a result, *Collinsella aerofaciens* (a predominant member of the phylum *Actinobacteria*) as well as two uncharacterized *Firmicutes* related to *Clostridium cocleatum* and *Coprococcus eutactus* were found to be significantly reduced in all IBS subtypes compared with the healthy controls. To further support the statistical differences observed, the same set of real-time PCR assays was applied to study another independent group of IBS patients (n=29). Again, highly similar results with similar statistically significant differences were obtained (150). In conclusion, the study showed that the fecal microbiota is indeed significantly altered in IBS patients.

The combined 16S rRNA gene composition in the fecal samples of 10 IBS-D patients was further analyzed by extending the analysis coverage from the three most variable %G+C fractions to the entire microbiota (158). The IBS-D 16S rRNA gene clone library was compared with an analogous healthy control library of 23 subjects. The study showed significant differences between clone libraries of IBS-D patients and healthy controls. The microbial communities of IBS-D patients were enriched in *Proteobacteria* and *Firmicutes*, but had depleted numbers of *Actinobacteria* and *Bacteroidetes* compared with the control. More specifically, 16S rRNA gene sequences belonging to the family *Lachnospiraceae* within the phylum *Firmicutes* were in greater abundance in the IBS-D clone library. Those authors therefore concluded that in future studies, the role of this family in IBS-D should receive more attention (158).

The study by Lyra et al. (179) investigated the fecal microbiota of 20 patients with IBS, including 8 IBS-D, 8 IBS-C and 4 IBS-M patients, in comparison with that of 15 healthy controls at three timepoints (0, 3 and 6 months of follow-up). To monitor the putative changes in the intestinal microbial communities, a set of 14 phylotype-specific real-time PCR assays was applied. Multivariate analysis of the phylotypes indicated that the intestinal microbiota of the patients with IBS-D not only differed significantly from the control group, but also from the other IBS subgroups over time. IBS-D patients presented significantly higher numbers of *Ruminococcus torques*-related phylotypes (179). Interestingly, in a very recent study in which correlations between self-reported symptoms of IBS and the GI microbiota composition were investigated, the abundance *R. torques*-related phylotype was associated with the severity of symptoms (188). The same phylotype was observed to remain abundant in the placebo group, but decreased significantly in the probiotic group, in a 6-month intervention trial with 42 IBS subjects (178). The abovementioned results suggest that this specific phylotype could be linked to IBS etiology. It is noteworthy that the phylogenetically most similar species, *R. torques*, is a known mucin degrader that has been shown to be elevated disproportionately to total mucosa-associated bacteria in epithelium in both CD and UC patients (243).

In a study by Swidsinski et al. (297), the composition and organization of the mucosal microbiota in biopsy specimens from unsubtyped IBS patients and healthy controls were investigated using fluorescent in situ hybridization (FISH) with a broad range of bacterial group-specific 16S rRNA gene-targeted oligonucleotide probes. The results showed that mucosal bacteria were more abundant in the samples of IBS patients than those of healthy controls. However, no differences in the proportional amounts of the predominant bacterial groups (e.g.


Review of the Literature

*Bacteroides-Prevotella, B. fragilis, E. rectale-C. coccoides, Faecalibacterium prausnitzii* and *Enterococcus faecalis* were observed between IBS patients and controls (297). The mucosal microbial composition of IBS patients was also evaluated in a study by Codling and co-workers (58) and compared with the fecal microbiota of the same subjects using a DGGE fingerprinting technique. The results showed that the average inter-individual similarity of the bacterial communities on the mucosa and in the lumen of IBS was not significantly different (58).

Overall, the data produced to date indicate that the intestinal microbiota is involved in the pathophysiology and symptom generation of IBS. However, the apparent question is whether the microbiota changes described are the effect, rather than the cause, of altered gut physiology. Further research is therefore needed to determine the contribution of gut physiology to intestinal microbiota in IBS. The application of whole-community metagenomics or functional genomics approaches on IBS cohorts would most likely yield potentially valuable information regarding the putative IBS-specific functions in the gut microbiota (261). Nevertheless, recent findings indicate that certain bacterial phylotypes are obviously associated with IBS and hence might serve as biomarkers in future clinical investigations. An excellent review by Salonen et al. (261) summarizes current research data regarding the role of GI microbiota in IBS.

2.3.6 Therapeutic modulation of intestinal microbiota in IBS

Numerous studies have attempted to alter the intestinal microbiota of IBS patients using different therapeutic approaches, including the use of probiotics, prebiotics, and symbiotics (20). The concept of probiotic (‘for life’) is over 100 years old now, since Elie Metchnikoff published ‘The prolongation of life: optimistic studies’ in 1907. In 1989 Roy Fuller defined probiotics as ‘Live microbial food supplements which beneficially affect the host by improving its intestinal microbial balance’ (100). More recently, this definition has been further refined to ‘Live microorganisms which when administered in adequate amounts confer a health benefit on the host’ (331). Probiotics have been applied and studied in a number of intestinal disorders such as *Clostridium difficile* colitis, antibiotic-associated diarrhea, IBD, and IBS. Probiotics possess beneficial mechanisms of action that include physical barrier effects against pathogenic bacteria, production of bactericidal proteins, and competition for nutrients. In addition, they have been demonstrated to improve the barrier function of the epithelium by stimulating mucin production in the colon and altering the immune activity of the host (49, 61, 227). The majority of commonly applied probiotics are lactic acid producing bacteria, which are able to decrease intestinal pH and thus suppress the growth of detrimental pathogenic bacteria. Probiotic microorganisms have also been shown to regulate the intestinal inflammatory response by modulating the secretion of cytokines and the immune response (227).

Numerous trials have evaluated the therapeutic effect of probiotics in IBS. This is justifiable, since probiotic administration holds great potential for providing benefits for intestinal fermentation or gas production, intestinal motility patterns, and microbiota-associated anti-inflammatory effects. In the majority of these trials single strains of lactobacilli or bifidobacteria have been applied, whereas less research has been carried out using multispecies probiotic combinations. In general, the results of the trials have been somewhat contradictory. This is due to heterogeneity in trial design, dosing regimen and species used. Moreover, several
trials have been hampered by considerable placebo effects, which is a common feature of IBS intervention trials (114, 222).

However, some of the recent well-designed probiotic intervention studies propose beneficial effects of probiotics over placebo on the alleviation of IBS symptoms, as well as on the pathophysiological features related to IBS. O’Mahony and co-workers (227) performed a double-blind, placebo-controlled study with 77 IBS patients randomized into three different treatment groups (Lactobacillus salivarius UCC4331, Bifidobacterium infantis 35624, or placebo) for a total of eight weeks. Bifidobacterium infantis but not L. salivarius strain improved the IBS symptoms of abdominal pain or discomfort, bloating or distension, as well as bowel movement difficulty, while increasing the serum IL-10/IL-12 ratio, suggesting a generalized anti-inflammatory effect (227). A larger multicenter double-blind, placebo-controlled intervention study by Whorwell and co-workers (332) was performed on 362 women diagnosed with IBS. The study subjects were randomly assigned to the groups receiving low or high doses of Bifidobacterium infantis 35624 or placebo, and were monitored for a total of four weeks. The results showed that there was an improvement in abdominal pain/discomfort that was 20% greater than with the placebo. In addition, an improvement in bloating/distension, sensation of incomplete evacuation, passage of gas, straining, and bowel habit satisfaction was observed. Interestingly, the benefits were only noted in the high-dose group receiving 1 x 10^{10} CFU per day of B. infantis (332).

Kim and co-workers (153, 154) performed two double-blind, placebo-controlled intervention trials that assessed VSL#3 in the treatment of IBS. VSL#3 is a combination of probiotics that contains live bacteria including Bifidobacterium (B. longum, B. infantis, and B. breve), Lactobacillus (L. acidophilus, L. casei, L. delbrueckii ssp. bulgaricus, and L. plantarum) and Streptococcus salivarius ssp. thermophilus. In the first study, 25 diarrhea-predominant IBS patients were included and randomly assigned to receive probiotics or placebo for eight weeks. In those who received the VSL#3 probiotic mixture there was no improvement in abdominal pain or colonic transit time, but a reduction in abdominal distension and flatulence was observed (153). The second study included 45 IBS patients whose main complaint was abdominal bloating/distension. The study participants received the probiotic mixture or placebo for 4-8 weeks. Compared with the placebo, VSL#3 led to a reduction in flatulence and a delay in colonic transit, but no improvement in abdominal pain was noted (154).

Another multispecies probiotic that has shown positive results in IBS is a mixture containing L. rhamnosus GG, L. rhamnosus LC705, B. breve Bb99, and Propionibacterium freudenreichii ssp. shermanii JS. A 6-month double-blind, placebo-controlled study by Kajander and co-workers (147) evaluated 103 IBS patients randomly assigned to a probiotic mixture or placebo treatment group. A 44% reduction in the symptom score (consisting of abdominal pain, distension, flatulence, and borborygm) was seen in the probiotic group at the end of the trial (147). These findings were confirmed by a 5-month follow-up study of 86 IBS patients in which the multispecies probiotic was shown to stabilize the intestinal microbiota, improve the quality of life, relieve abdominal pain, and reduce constipation, with no noticeable adverse effects (148).

Prebiotics are non-digestible food ingredients that stimulate the growth and/or activity of bacteria in the digestive system that are beneficial to the health of the body (105). Only a few studies have examined prebiotics as a treatment strategy for IBS. This
is most likely due to the fact that the inclusion of fermentation substrates could worsen intestinal gas production in people suffering from IBS (244). A small 4-week double-blind, cross-over trial of daily fructo-oligosaccharide (FOS) supplementation in 21 patients with IBS (both IBS-D & IBS-C) showed no effect on symptom scores, whole gut transit time, fecal weight or fasting breath hydrogen (142). However, a more recent intervention trial comparing 5 g FOS with a placebo in 104 patients with mild functional bowel disease found that those in the study group had a significant decrease in the intensity of their symptoms using a visual analogue score (232). However, that study encompassed patients with varying types of functional bowel disorders and no subgroup analysis was performed. Nevertheless, the promising results with prebiotics have encouraged the application of approaches based on symbiotics (a combination of pro- and prebiotics) for the management of IBS. A parallel 12-week group study comparing *L. paracasei* (strain B21060) in conjunction with a prebiotic mixture versus the prebiotic mixture alone in 267 patients with IBS showed that the symbiotic treatment significantly increased the number of patients who rated their pain as absent or mild compared with the baseline. In addition, a subgroup analysis within the IBS-D population revealed a considerable reduction in bowel movements and abdominal pain in the symbiotic treatment group (5).

Although understanding of the GI tract microbiota continues to expand and its involvement in the pathogenesis of IBS has been addressed, IBS remains a difficult condition to treat. This is mainly due to the heterogeneous nature of the syndrome. Therefore, future research should focus on targeted treatments for different subtypes of IBS, since it is unlikely that all patients with IBS have the same underlying etiology. However, as the abovementioned intervention studies have demonstrated, manipulation of the GI microbiota offers therapeutic benefits in the alleviation of IBS symptoms, at least in a proportion of patients. This provides further evidence regarding the essential role of the intestinal microbiota in IBS.
3. AIMS OF THE STUDY

The overall aim of the present study was to develop real-time PCR-based applications for the analysis of fecal bacterial populations, in order to uncover putative alterations in the intestinal microbial community structure of people suffering from IBS.

Specific objectives in the respective studies were:
1. To design and optimize an extensive set of 16S rRNA gene-targeted species- and group-specific assays for accurate quantification of bacteria from fecal samples with real-time PCR (Study I).
2. To design and optimize a real-time PCR intestinal pathogen panel and examine the putative involvement of selected of intestinal pathogenic bacteria in IBS (Study IV).
3. To compare the intestinal microbiota of patients suffering from IBS with a healthy control group devoid of gastrointestinal (GI) symptoms (Study II).
4. To investigate the mode of action of a multispecies probiotic consisting of Lactobacillus rhamnosus GG, Lactobacillus rhamnosus Lc705, Propionibacterium freudenreichii ssp. shermanii JS, and Bifidobacterium breve Bb99 by monitoring its effects on intestinal microbiota and markers of microbial fermentation activity (Study III).

![Diagram](image)

Figure 9. Outline of the protocol pipeline of this study. The primer in silico design and optimization tasks are reported in I and IV, while the majority of fecal sample analyses are included in II, III, IV.
4. MATERIALS AND METHODS

4.1 STUDY SUBJECTS

4.1.1 IBS patients (I-IV)
Subjects fulfilling the Rome I (Study IV) or II (Studies I-IV) criteria for IBS were recruited from primary care units (Helsinki and Kuopio, Finland) by experienced physicians. These IBS patients were predominantly female. The study subjects fulfilling the Rome I criteria were 20-72 years of age and their general condition was confirmed as good by medical experts. Exclusion criteria for participation included presence of organic GI diseases, inadequately treated hypertension or pharmacologically treated diabetes. Use of statins, pharmacologically treated hypertension or coronary artery disease were not considered exclusion criteria if medication had been used for at least six months prior to the study with no changes in dosage. The patients fulfilling the Rome II criteria were 20-65 years of age and had undergone clinical investigation and endoscopy or barium enema of the GI tract within the year prior to the study. Exclusion criteria were pregnancy, breast feeding, organic intestinal diseases or other severe systematic diseases, previous major or complicated abdominal surgery, severe endometriosis, antimicrobial medication during the previous two months, and dementia or otherwise inadequate cooperation. Patients with lactose intolerance were included if they reported following a low-lactose or lactose-free diet. Some of the patients were receiving medication for their IBS symptoms, and were allowed to continue their medication throughout the study.

4.1.2 Healthy control subjects (I, II, IV)
Healthy control subjects devoid of GI symptoms were recruited to form an age- and gender-matched control group for the IBS patients. The recruitment of a control group devoid of regular GI symptoms was coordinated by VTT Biotechnology (Espoo, Finland). The inclusion criteria for the control subjects were overall good health and aged between 20 and 65 years. Intestinal disturbances (including lactose intolerance and celiac disease) and ongoing antibiotic treatments were considered exclusion criteria for the control group.

4.2 STUDY DESIGN OF THE PROBIOTIC INTERVENTION
The probiotic intervention in Study III was performed in a randomized, double-blind, placebo-controlled manner with two parallel groups. A total of 55 IBS patients (Rome II criteria) participated in the study. The subjects were randomly assigned into probiotic (n=28) or placebo (n=27) group and advised to follow their usual dietary habits and not make any changes to possible medication (e.g., fiber analogues, antispasmodics, antidiarrheals, laxatives). Any changes in medication, health status or dietary habits as well as antimicrobials and adverse events were recorded. Consumption of other probiotic products was not allowed during the study.

During the 6-month study period, each subject received daily either a probiotic capsule (Valio Ltd, Helsinki, Finland) containing *L. rhamnosus* GG (ATCC 53103, LGG), *L. rhamnosus* Lc705 (DSM 7061, Lc705), *P. freudenreichii* spp. *shermanii* JS (DSM 7067, PJ5) and *B. breve* Bb99 (DSM 13692, Bb99) or a placebo capsule. The total daily amount of bacteria was 8–9 × 10⁹ CFU per day, with equal amounts of each strain.
4.3 COLLECTION OF FECAL SAMPLES
Fecal samples were collected from IBS patients (Rome II criteria) in I-IV and from healthy control subjects in Studies I, II, and IV. For II and III, the fecal samples were obtained on three occasions at three-month intervals (baseline, 3 and 6 months). A small subset of baseline samples was applied in Study I. In addition to the abovementioned baseline samples, an additional set of fecal samples of IBS patients (Rome I criteria) was collected to be analyzed in Study IV. All samples from IBS and healthy control subjects were stored immediately after defecation in plastic anaerobic containers, aliquoted and stored at -80°C prior to analysis.

4.4 INTESTINAL MICROBIOTA ANALYSIS OF IBS PATIENTS AND CONTROLS
The intestinal microbiota analyses of IBS patients and healthy controls were carried out in all studies by real-time PCR. In Study II, the fecal microbiota of IBS patients (n=27; placebo group of the probiotic intervention study) was compared with age- and gender-matched control subjects (n=22). Fecal samples were obtained at 3-month intervals (baseline, 3 and 6 months) and were analyzed with a total of 20 species-, genus- or group-specific real-time PCR assays targeting the 16S rRNA gene (the majority designed and validated in Study I; see Section 4.5) with overall coverage of approximately 300 known bacterial species. The assays in question were selected mainly based on a priori indications of an association with IBS or due to their predominant nature in the lower intestinal microbial community.

In Study III, the fecal samples from three sampling points of the IBS patients (the probiotic group) participating in the probiotic intervention study were subjected to real-time PCR analyses using principally the same set of species-, genus- or group-specific real-time PCR assays as in Study II. Moreover, previously published strain-specific real-time PCR assays were applied for all three samples of each participant to monitor each probiotic species administered (118, 210). The probiotic real-time PCR analyses and the biochemical analysis (concentration of fecal SCFAs and bacterial enzyme activity) were carried out elsewhere.

The rationale of Study IV was to determine the putative involvement of selected intestinal pathogenic bacteria in IBS. For this, the abovementioned control and IBS samples (baseline) were subjected to real-time PCR analysis in conjunction with an additional set of fecal samples of IBS patients fulfilling the Rome I criteria (n=45). Overall, a total of 12 real-time PCR assays targeting the virulence-related or 16S rRNA genes of the pathogenic bacteria were applied for analysis of the complete set of baseline samples.

4.5 MOLECULAR MICROBIOLOGICAL AND BIOCHEMICAL TECHNIQUES
4.5.1 DNA extraction for real-time PCR analyses (I-IV)
Rapid isolation of genomic DNA from pure cultured bacteria (positive and negative controls) was carried out by mechanical bead beating disruption of bacterial samples in the presence of glass beads, followed by phenol-chloroform extraction and ethanol precipitation. The microbial strains used in this study have been described in detail in the respective original publications I-IV. A large-scale bacterial DNA isolation from fecal samples was performed according to Apajalahti et al. (9) to produce a sufficient amount of high-quality template DNA for real-time PCR. Fecal bacteria were first washed with repeated low-speed centrifugations to remove the undigested particles.
Subsequently, the bacteria were pelleted by high-speed centrifugation from the combined supernatants, after which the purified cell pellets were lyzed with a combination of freeze-thaw cycles, lysozyme and vortexing with glass beads. The concentration of the extracted DNA was determined by a fluorometric method based on the fluorochrome Hoechst 33258 using VersaFluor Fluorometer System.

In order to evaluate the reliability and efficacy of the DNA extraction method, _E. coli_ subgroup-specific 16S rRNA gene primers (187) were applied for quantification of _E. coli_ genomes from serial dilutions of overnight cultured _E. coli_ DSM 6897 with real-time PCR. Cell density of the overnight culture was estimated by viable count on Luria agar and microscopic cell count with the Petroff Hauser Counting Chamber (Hauser Scientific Company, USA).

4.5.2 Design of real-time PCR assays (I, IV)

SYBR Green I-based bacterial species-specific or group-specific oligonucleotide primers for real-time PCR analyses were designed and/or validated by employing the published sequence data available in DNA sequence databanks (I, IV). Initially, published 16S rRNA gene sequences were extracted from databases and target sequences with a likely incorrect annotation (shown by low match scores to other sequences of the same species) with unexpected deletions/insertions were excluded to avoid misleading alignments. Subsequently, multiple alignments of the target sequences with those of related bacterial reference strains were constructed with the program Clustal W (303) provided by the European Bioinformatics Institute. Potential primer candidates specific for the targets of interest were assessed either manually or applying Primer3 primer design online interface (258) provided by the Whitehead Institute. Secondary structure analyses of the target templates were executed with the mfold DNA-folding server (349). Finally, the desired specificity of the primer pairs towards intended target DNA was verified by submitting the sequences to the Basic Local Alignment Search Tool (BLAST) of NCBI National Center for Biotechnology Information (NCBI) (2), the FASTA3 database search program of the European Bioinformatics Institute (EBI) (237) and/or the Probe Match program provided by the Ribosomal Database Project (RDP II) (59). In addition, signature oligonucleotides previously published by others were utilized or modified when necessary (I, II, IV). The complete set of real-time PCR assays, their primer sequences and annealing temperatures applied in this study are listed in Table 4, while the target bacterial species of each assay are described in detail in the respective original publications.

4.5.3 Assay optimization (I, IV) and amplification conditions (I-IV)

End-point PCR (I, IV)
The optimal annealing temperatures of the PCR primer pairs and the correct product sizes were determined with end-point gradient PCR (Peltier Thermal Cycler PTC-200; MJ Research, USA). The amplification reactions were carried out in a total volume of 50 µl, and the standard reaction mixture consisted of 10 mM Tris-HCl (pH 8.8), 150 mM KCl, 0.1% Triton X-100, 1.5 mm MgCl₂, 200 µM each dNTP, 1 µM of each primer, and 0.024 U/µl Dynazyme II Taq polymerase (Finnzymes, Finland). The amplification program included an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 15 s, primer annealing at 50-70°C gradient for 20 s and primer extension at 72°C for 45 s, with a final extension step at 72°C
<table>
<thead>
<tr>
<th>PCR assay</th>
<th>Target gene</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Amplicon length (bp)</th>
<th>MgCl₂ (mM)</th>
<th>Tₘ (°C)</th>
<th>Substudy</th>
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<td>3</td>
<td>64</td>
<td>IV</td>
</tr>
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<td>F: GGTGTCCGTTCAGTGCTGACAT R: CGACGGTTCACCGCTCCA</td>
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<td>68</td>
<td>I, II, III</td>
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<td>58</td>
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<td>Amplicon length (bp)</td>
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<td>Tₘ (°C)</td>
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<td>3</td>
<td>55</td>
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Table 4 continuing

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<th>MgCl₂ (mM)</th>
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<th>Substudy</th>
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<td><em>iap</em></td>
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<td><em>Yersinia enterocolitica</em></td>
<td><em>ail</em></td>
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<td><em>S</em>-nuclease probe</td>
<td><em>16S rRNA</em></td>
<td>F: GGTCTAGGTGATTGTTGATTACTATAC</td>
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</table>

*TaqMan assay applied with *S*-nuclease probe.
for 5 min. The specificity of the primer pairs was confirmed by employing pure culture DNA of an extensive set of predominant and pathogenic GI tract bacterial species as negative controls. The PCR products were subjected to electrophoresis on agarose gels and stained with ethidium bromide to determine optimal annealing temperature and verify the correct amplicon size.

**Real-time PCR (I-IV)**

In general, real-time PCR amplifications were performed using an iCycler iQ Real-Time Detection System (Bio-Rad, USA) associated with the iCycler Optical System Interface software (version 2.3; Bio-Rad) using SYBR Green I (I-IV) or TaqMan (II, III) chemistry. All real-time PCR experiments were carried out in triplicate with a reaction volume of 25 μl, using iCycler IQ 96-well optical grade PCR plates (Bio-Rad, USA) covered with iCycler optical-quality sealing film (Bio-Rad, USA).

In Studies I and IV, optimal efficiency of PCR amplification was assessed for each candidate primer pair by testing various MgCl₂ concentrations with a dilution series of genomic DNA from the target test species. The optimized reaction conditions for each PCR assay applied in this thesis are summarized in Table 4.

The reaction mixtures for the optimized SYBR Green I-based assays consisted of a 1:75 000 dilution of SYBR Green I (Molecular Probes, USA), 10 mM Tris-HCl (pH 8.8), 150 mM KCl, 0.1% Triton X-100, 2-5 mM MgCl₂ (assay dependent), 100 μM of each dNTP, 0.5 μM of each primer, 0.02 U/μl II Taq polymerase (Finnzymes, Finland), and either 5 μl of template or molecular grade water as no-template control (NTC). Amplification involved one cycle at 95°C for 5 min for initial denaturation, followed by 35 cycles of denaturation at 95°C for 15 s, primer annealing at the optimal temperatures (see Table 1) for 20 s, extension at 72°C for 30 s and an additional incubation step at 80-85°C for 30 s to collect the fluorescent data. An extensive set of representative GI bacterial species was used as negative controls. To determine the specificity of PCR reactions, melt curve analysis was carried out after amplification by slow cooling from 95 to 60°C, with fluorescence collection at 0.3°C intervals and a hold of 10 s at each decrement.

The reaction mixtures of 5’-nuclease (TaqMan) assays consisted of 10 mM Tris-HCl (pH 8.8), 150 mM KCl, 0.1% Triton X-100, 2-4 mM MgCl₂, 200 μM of each dNTP, 1 μM of each primer, 80 nM of fluorescent probe, 0.03 U/μl Dynazyme II (Finnzymes, Finland), and either 5 μl template or molecular grade water as no-template control (NTC). An initial denaturation at 95°C was followed by 30-40 cycles of denaturation at 95°C for 15 s, and a combined incubation step for primer annealing and extension, during which the fluorescent signal was also measured. An extensive set of representative GI bacterial species was used as negative controls. The TaqMan assays applied in this study were previously described by Malinen et al. (187) and Matsuki et al. (200) (II); and Halme et al. (118) and Mikkola et al. (210) (III).

**4.5.4 Preparation of genomic DNA standards (I-IV)**

For construction of standard curves, 10-fold dilution series of between 0.1 pg and 10 ng (approx. 30-100 to 3.0 × 10⁻¹ to 1.0 × 10⁰ target genomes, depending on the genome size) from target species genomic DNA preparations in conjunction with 25 ng and 250 ng of each fecal DNA preparation on each 96-well plate were applied for real-time PCR. The standard curves of individual real-time PCR assays were used for quantification of the target bacterial DNA from fecal DNA preparations. The cycle number at which the fluorescence passes the manually set threshold was determined
for the sample DNA and compared with the standard curves.

4.5.5 Real-time PCR data interpretation (I-IV)
For each individual assay, real-time PCR results were converted to the average estimate of target bacterial genomic equivalents present per gram of feces (wet weight). For the calculations, the original mass of starting material, estimated average genome size of target organisms, bacterial DNA yield, elution volume, and PCR template dilution were taken into account. The genome sizes applied were 2.3 Mb for Lactobacillus spp. (I, II, III), 2 Mb for Bifidobacterium spp. (I, II, III), 3 Mb for Enterococcus spp. (I, II, III), 4 Mb for C. coccoides group (I, II, III), 2.2 Mb for Fusobacterium spp. (I), 3 Mb for Veillonella spp. (I, II, III), 3 Mb for C. perfringens group (I, II, III), 3 Mb for Desulfovibrio spp. (I, II, III), 3 Mb for Atopobium group (I, II, III), 4 Mb for Bacteroides-Prevotella-Porphyromonas group (I, II, III), 4.6 Mb for E. coli subgroup (II, III), 5.2 Mb for B. fragilis (II, III), 4.5 Mb for Aeromonas spp. (IV), 5.4 Mb for B. cereus group (IV), 1.6 Mb for C. jejuni (IV), 4 Mb for C. difficile (I, II, IV), 3.2 Mb for Cl. perfringens (IV), 5.4 Mb for EHEC and EPEC (IV), 1.7 Mb for H. pylori (I, IV), 2.9 Mb for L. monocytogenes (IV), 4.8 Mb for Salmonella spp. (IV), 3.5 Mb for S. aureus (IV), and 4.8 Mb for Y. enterococccica (IV). The amounts of F. prausnitzii genomes in fecal samples were estimated assuming that the 16S rRNA gene copy number of the species in question was 10. In 16S rRNA gene-targeted assays the differences in the 16S rRNA gene copy numbers were omitted due to the fact that the majority of such assays were set to detect several bacterial species, most likely with varying rRNA gene copy numbers.

4.5.6 Sequencing of selected PCR products (II, IV)
In addition to real-time PCR targeting Campylobacter spp. (II) and Staphylococcus aureus (IV), the real-time PCR amplicons were sequenced to verify the identity of the gene fragments obtained. PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen, Germany) after excising from 1.25% SeaPlaque agar (Cambrex, USA), and eluted in 35 µl of elution buffer. Subsequently, the concentration of the purified amplicons was estimated with serially diluted samples on 0.8% agarose gels with ethidium bromide staining. The sequencing was carried out with ABI 310 Genetic Analyzer (Applied Biosystems, USA) using the Big Dye Terminator chemistry (Applied Biosystems, USA). Two sequencing reactions were performed from individual PCR products with Campylobacter spp.-specific and S. aureus-specific forward and reverse primers, respectively. Finally, the sequences were processed with Sequencer™ 3.0 sequence analysis software (Gene Codes Corporation, USA) and a database search was performed against the GeneBank sequences.

4.5.7 Analysis of selected intestinal metabolites (III)
Fecal SCFA content (acetate, butyrate, propionate, valerate, caproate, isobutyrate, isovalerate, iso-caproate) was identified with gas chromatography according to the method described by Høverstad and co-workers (137). Bacterial enzymes (β-glucosidase and β-glucuronidase) were analyzed with a method described by Goldin et al. (108). Enzyme activity was expressed per gram of protein, and the protein content was determined with a protein test kit (Bio-Rad Protein Assay, Bio-Rad, USA).
4.6 STATISTICAL ANALYSIS
In Study II, the results are illustrated as means with 95% confidence intervals (CI) or as medians with interquartile ranges. Comparisons between different IBS subgroups and between IBS patients and healthy controls were performed by the Kruskal-Wallis signed rank test and the Mann-Whitney U test, respectively. In Study III, the main results are presented as means with 95% CI or with the standard error of mean. Analysis of covariance with baseline as covariate was used for comparisons between the probiotic group and the placebo group concerning the main endpoints, IBS symptoms and microbiota. In Study IV, for statistical comparison, Pearson’s chi square test was performed to examine correlations between the presence/absence results of pathogen detection by PCR and the different subject groups. In addition, the R software environment for statistical computing and graphics (247) was used for the data illustration.

4.7 ETHICAL ISSUES
The study was approved by the Human Ethics Committee at the Joint Authority for the Hospital District of Helsinki and Uusimaa (HUS) (I-IV) and Kuopio University Hospital Ethical Committee for Research (IV) for the IBS patients, and by the ethical committee of VTT, Finland, for the controls (I, II, IV). Written informed consent was obtained from each participating subject. Participation in the study was voluntary and the patients were allowed to withdraw at any point without explanation. The abovementioned samples have been analyzed in multiple studies under the Finnish Funding Agency for Technology and Innovation (TEKES), project (no. 40039/03).
5. RESULTS AND DISCUSSION

5.1 GENERAL OPTIMIZATION OF SYBR GREEN I-BASED PCR ASSAYS (I, IV)

A total of 15 novel real-time PCR assays for quantifying endogenous, pathogenic or potentially IBS-associated bacteria from human intestinal samples were designed and validated in this study. In addition, previously published primers for the *Lactobacillus* group (I, II, III), selected pathogens (IV), and eubacteria (II, III) were optimized and validated, since they were not originally designed for SYBR Green I-based applications.

5.1.1 Sensitivity and linearity of the assays

The dynamic range of the SYBR Green I-based assays was examined using 10-fold serial dilutions of target DNA extracted from pure cultures of target bacteria. The linearity of the standard curve was determined for the range from 0.1-10 pg to 10 000 pg of specific target genomic DNA (a dynamic range of 4 to 5 logs), which corresponds to 30-4500 to 1.9 × 10⁶ -6.0 × 10⁸ target genomes per PCR reaction, depending on the genome size of a target bacterium. Thus, considering the dilution factor for DNA extraction, the detection limit for the assays with best performance was approximately 3 × 10⁴ target bacteria per gram of fecal matter. It is noteworthy that the feasible upper limit of the standard curve was not determined and could actually be higher, further increasing the dynamic range of the assays. The results obtained from artificial pure culture DNA samples were confirmed by a reconstruction assay as described below.

The amplification efficiency for the assays with pure culture target DNA was obtained by plotting the threshold cycle values against starting DNA quantity. Using the formula \( E = 10^{(-1/\text{slope})} - 1 \), the efficiency of the individual assays was between 78.3 and 103.5%. Melt curve analysis was carried out in conjunction with each real-time PCR assay to distinguish the fluorescent signal obtained from the specific amplification product from artefacts such as primer-dimers. The melting temperature (T_m) of the desired PCR amplicons varied between 81 and 90°C, whereas primer-dimers with considerably lower T_m values were observed with no-template and negative controls, as well as with lower template concentrations in certain assays.

5.1.2 Specificity of the assays

Assay specificity is a key factor for accurate quantification of bacteria of interest from clinical samples. To decrease the putative cross-reactivity of the assays with unwanted DNA, the amplification conditions of PCR were optimized to be exceedingly stringent. For this, gradient end-point PCR was used to select the optimal primer annealing temperatures for each assay. The specificity of the primer pairs was subsequently tested against an extensive collection of intestinal bacterial species in real-time PCR with optimized reaction conditions for each assay. Only weak signals due to background fluorescence were observed with any of the non-target microorganisms tested. The assay specificity was further confirmed with agarose gel electrophoresis in which PCR products of the expected sizes were obtained, and no additional bands were observed.

Although the assays validated and applied in this study were shown to be specific for the target organisms both *in silico* and *in vitro*, any oligonucleotide primer or probe signature should be considered specific only in relation to the current sequence data. However, the continuously increasing sequence data submitted to the public databases is shedding light on the true diversity of intestinal microbiota,
while also facilitating the design of primers with optimal specificity for the given target organisms. An important approach to verify the assay specificity is the sequencing of the PCR products obtained from various sample sources (e.g., intestinal samples of several individuals, samples derived from different compartments of the GI tract or intestinal samples from species). In this study, sequencing of PCR products was not performed in the assay validations for economic reasons.

The rationale to use 16S rRNA gene as a target to design the real-time PCR primers for intestinal bacterial groups or genera was that 16S rRNA gene is the most extensively studied gene fragment within prokaryotic microorganisms, with huge amounts of sequence data available in the public databases. This facilitated the identification of sequences exclusive to our target species, as discussed above. On the other hand, the major disadvantage of this 16S rRNA gene approach in the design of species-specific primers is the fact that the variable regions within the gene can be almost identical for closely related bacteria. Therefore, to minimize the possibility of unspecific cross-reactions, we applied primers targeting the highly specific virulence genes in the intestinal pathogens selected for analysis (IV).

5.2 APPLICATION OF REAL-TIME PCR FOR FECAL BACTERIAL ANALYSIS (I)
In real-time PCR approaches of intestinal samples, the initial step is always the DNA extraction and purification. Therefore, an efficient technique for the recovery of bacterial DNA is essential for successful molecular analysis of complex microbiota. If the isolated community DNA does not accurately represent the microbial composition of the original sample due to inefficient cell disruption or inadequate DNA purification procedure, the quantitative results of the real-time PCR analyses will be unreliable and the resulting bias can be even greater than in conventional culturing methods.

To ascertain that the real-time PCR in conjunction with the DNA extraction method of fecal bacteria described in this study were able to provide reliable quantitative results with feasible sensitivity, a set of fecal samples was subjected to reconstruction assays. This was done by spiking two replicate fecal specimens with 10-fold serial dilutions from pure cultures of H. pylori, C. difficile, and C. jejuni. Bacterial densities in the pure cultures were estimated microscopically and adjusted to the required dilutions between $6 \times 10^8$ and $6 \times 10^9$ cells per gram of fecal specimen. Due to the microscopic counting, a fairly accurate reference number of cells was obtained and, similarly to PCR, made no distinction between living and dead bacterial cells.

The results of the spiking assay are shown in Figure 10. Overall, an appropriate linearity was obtained with all assays tested. However, the best linear range was obtained with H. pylori-specific PCR, which gave an accurate quantitative outcome from the fecal preparations with each dilution applied ($6 \times 10^8$ to $6 \times 10^9$). With C. difficile, inclusion of $6 \times 10^4$ target cells could still be quantified, whereas C. jejuni was quantifiable only between $6 \times 10^6$ and $6 \times 10^7$ added cells g$^{-1}$ feces. Beyond that limit, the presence of the target organisms in the samples can still be detected using melting curve verification, but the results will not be accurately quantitative. It is likely that the deceased sensitivity with the latter two assays was at least partly due to primer-dimer -derived background fluorescence, which is a typical phenomenon in SYBR Green I-based assays. We have previously shown that the use of hot-start polymerase in the SYBR Green I-based real-time PCR procedure reduces the formation
of primer-dimers, thus improving assay sensitivity (187). However, for economic reasons hot-start enzymes were not employed in this study. Considering the inaccuracies that are likely to be introduced into the results by sample processing and concentration measurement of fecal DNA preparations, the observed level of accuracy and sensitivity for the quantification of target genomes was regarded as satisfactory.

In addition to the reconstruction analysis of fecal samples, a serial dilution from overnight cultured E. coli was analyzed with real-time PCR to further verify the accuracy of the technique. Viable count and microscopic calculation were applied for quantification of the E. coli cells from the overnight culture. The real-time PCR gave slightly higher values than the viable counts and a linear range of quantification between $6.5 \times 10^6$ and $6.5 \times 10^7$ target cells was obtained. This was most likely due to the fact that in addition to the viable cells, PCR applications also detect dead cells present in bacterial cultures.

In conclusion, reconstruction assays in which fecal samples were spiked with various amounts of target cells confirmed the feasibility of real-time PCR for quantitative evaluation of intestinal bacteria from human feces. Moreover, good recovery rates of the added bacteria from feces further confirmed the efficiency of the DNA isolation method developed by Apajalahti et al. (9). This was also recently demonstrated in a comparative study in which four widely used DNA extraction protocols were evaluated (262).

In addition to efficient DNA recovery, the protocol yielded high-quality DNA, which was established by the fact that increasing the total DNA template amount subjected to real-time PCR from 50 to 250 ng further improved

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**Figure 10. Detection of specific target DNA in a spiking assay.** Approximately $6 \times 10^6$, $6 \times 10^7$, $6 \times 10^8$, $6 \times 10^9$, or $6 \times 10^3$ target bacterial cells introduced into replicate fecal samples (open and closed squares) prior to the collection of bacterial cells and isolation of DNA. Three parallel PCR reactions were analyzed. Results are shown for A) *H. pylori*, B) *C. difficile* and C) *C. jejuni* as mean amounts and standard deviations of target genomes in one gram of feces. Copyright: The Society of Applied Microbiology, Journal of Applied Microbiology.
the detection limit of the assays, without the interference of inhibitory factors. This facilitates the detection and quantification of minor subpopulations in feces. An apparent challenge of real-time PCR with primers designed to target a wide range of phylogenetically related or unrelated bacteria is unpredictable variation in the number of 16S rRNA gene operon copy numbers especially in the case of noncultivable species for which only the 16S rRNA gene is known. Therefore, the differences in 16S operon copy numbers within the targeted bacterial genera or groups were omitted in this study. However, the probability of bacterial species possessing the same number of operons increases with decreasing phylogenetic distance. Hence, the utilization of chromosomal DNA as a standard was reasonable in order to diminish errors in data analysis.

5.3 INTestinal microBiotA in IBs PatienTs and healthy controls (II)

IBS is a common multifactorial functional intestinal disorder, which pathogenesis is not completely understood. As discussed above, growing amount of scientific evidence suggests that microbes of complex intestinal ecosystem are involved in the onset and maintenance of IBS. Therefore, the GI microbiota of 27 IBS patients and 22 healthy volunteers was analyzed using a set of 20 qPCR assays covering approximately 300 bacterial species. Moreover, to shed light on the temporal alterations of the microbiota, the samples from each participant were collected in three different time points at three months intervals.

The results showed considerable variation in the microbiota composition on an individual level among both IBS and control groups. Nevertheless, when the different subgroups of patients were compared, lower numbers of *Lactobacillus* spp. were present in the samples of diarrhea-predominant IBS patients compared with constipation-predominant IBS patients (p=0.019). Furthermore, in comparison with the healthy controls, the constipation-predominant IBS patients harbored increased amounts of *Veillonella* spp. (p=0.045). With respect to the typical microbiota of IBS patients during the 6-month follow-up period, real-time PCR also identified a significant decrease in the *Bifidobacterium catenulatum* group (p=0.039) and *Clostridium cocoides* subgroup (p=0.003) between IBS patients and healthy controls. Other group-level assays did not show significant differences between the subject groups. At species level, *Ruminococcus productus - Clostridium cocoides* was significantly increased in all IBS subtypes compared with the healthy controls (p=0.04).

The presence of *Campylobacter* spp., *Helicobacter* spp., and *C. difficile* in the feces of IBS and control subjects was also analyzed. No indications of the presence of *Helicobacter* spp. or *C. difficile* were found in either of the subject groups. *Campylobacter* spp. was found to occur in the samples of five IBS patients, whereas no positive cases were observed among control subjects. Sequencing of the PCR products obtained from *Campylobacter* genus PCR confirmed this finding. However, a single IBS subject diagnosed with an alternating type IBS harbored *Campylobacter jejuni*, whereas the other four patients with indications of *Campylobacter* spp. harbored either commensal (*C. hominis*) or oral campylobacteria.

To our knowledge, the Study II was the first to address the putative quantitative microbiota differences in IBS patients by the application of real-time PCR. Although the subject numbers were relatively small, the lack of preceding research makes this study a significant work in the intestinal microbiota characterization of IBS patients with nucleic
Results and Discussion

Acid-based techniques. Comparisons with the results of previous conventional culture-based studies concerning alterations of the fecal microbiota in IBS have to be made with caution due to various limitations of these studies (235), as well as the entirely different analytical techniques applied (culturing vs. real-time PCR). However, in the study by Balsari et al. (18), lower concentrations of lactobacilli and bifidobacteria were detected in patients with IBS. Hence, these results are to some extent in agreement with our observations, although unlike Balsari et al. we did not observe differences in total Bifidobacterium spp. between the IBS patients and healthy controls. However, differences were observed in the B. catenulatum group when the pooled results from three fecal samples of 21 patients and 15 healthy controls were analyzed. Interestingly, a highly significant decrease in the levels of B. catenulatum in the fecal samples of IBS patients was also reported in a recent real-time PCR-based study with 41 IBS patients and 26 healthy subjects by Kerckhoffs and co-workers (152), thus supporting our findings. The impact of B. catenulatum on the human intestinal health is unknown. It is likely that the lower levels of this bacterial species might develop as a result of altered gastrointestinal motility or genetic makeup of IBS patients rather than being the underlying cause of IBS symptoms (152).

In contrast to our study, Tana et al. (299) showed increased numbers of Lactobacillus in IBS patients using real-time PCR. This conflicting result is possibly explained by the differences in the recruiting criteria of the study subjects and experimental design (e.g., three vs. one sample collection points). Different dietary profiles might also be associated with the discrepancy. Interestingly, fecal samples from IBS patients exhibited higher counts of Veillonella spp., which is compatible with our study. Moreover, irritable bowel syndrome patients expressed significantly higher levels of acetic acid, propionic acid, and total organic acids than controls (299). As the members of genus Veillonella are known to convert lactic acid into acetic and propionic acid (84, 270), the obtained results strongly suggest that increased Veillonella levels result in the production of higher levels of acetic and propionic acid from intestinal lumen carbohydrates in IBS patients.

In conclusion, Study II provides evidence and supports the earlier suggestions of a potential role of altered intestinal microbiota in the pathogenesis of IBS. Fecal microbiota of the IBS subgroups appear to differ from one to the other, which is to be expected considering the different kinds of symptoms involved in the disorder. It is evident that a large proportion of intestinal bacteria is as yet uncharacterized and evaded the PCR analyses applied in this study. However, this work provides an important basis for the application of real-time PCR in analysis of intestinal bacteria in health and disease.

5.4 Effect of Probiotic Supplementation on the Gut Microbiota in IBS Patients (III)

In Study III, the recovery of three probiotic strains administered and the abundance of 17 intestinal bacterial groups or species were monitored with real-time PCR. The target bacterial groups were selected according to the Study II, which indicated an association between IBS and the bacteria in question, or due to the predominant nature of the group in the gut ecosystem. The probiotic strains LGG and Bb99 were detected in the majority of the subjects in the probiotic group after 6 months of supplementation. The prevalence rates in this group at the 6-month sampling points were 95% for LGG and 79% for Bb99. On the other hand, the number of subjects
Results and Discussion

in the probiotic group harboring Lc705 and PJS during the intervention was lower than that carrying LGG and Bb99. Lc705 was found in 53% of the subjects at the 6-month sampling point, while PJS was detected in 63% at 6 months. One possible reason for the somewhat low prevalence was the inability of the strains to remain viable in the GI tract. Orally administered probiotic strains must retain the ability to survive the manufacturing process, transit through the stomach and small intestine, tolerate acidic conditions, survive and grow in the presence of bile and colonize the human gastrointestinal tract. It is likely that especially in the case of Lc705 and PJS, these criteria were not achieved, and therefore the strains were autolysed and remained undetected in some study subjects.

Surprisingly, LGG was found in 43% and 24% of subjects in the placebo group at 3 and 6 months, respectively, and PJS was detected in two of 21 (10%) of the samples at both 3 and 6 months. Moreover, these strains were also detected in a number of samples within the placebo group at baseline. This observation may reflect the wide commercial availability and exceptionally high consumption of probiotic products in Finland. Another explanation for this unexpected result was the putative cross-reaction of the strain-specific assays designed with endogenous strains belonging to the species in question.

The intestinal microbiota remained stable during the intervention in both treatment groups. The exception was *Bifidobacterium* spp., the numbers of which (as log10) increased in the placebo group (from 9.2 at baseline to 9.8 at 6 months), while the numbers decreased in the probiotic group (from 9.6 to 9.1 in the same period). The observed difference between the two study groups was statistically significant (p=0.028). The reduction in *Bifidobacterium* spp. in the probiotic group and increase in the placebo group remain unclear. It appeared that only those with originally high counts of *Bifidobacterium* spp. suffered the decrease. Therefore, it is possible that individuals harboring significant levels may be more susceptible to temporal variations in bacterial counts or that competitive inhibition between the supplemented and endogenous bifidobacteria has taken place.

It is noteworthy that regardless of the lack of significant impact on the intestinal bacterial target groups analyzed, the probiotic mixture appeared to be effective in alleviating IBS symptoms (147). Hence, the results of this study are not in accordance with the claim that one of the favorable effects of probiotics in IBS is attributable to modulation of the microbiota and its metabolism. It should be borne in mind, however, that the human intestine is estimated to be colonized by a complex microbiota comprising more than 1000 species (250) and our study included analyses on 20 bacterial species or groups. Therefore, it is possible that certain relevant, yet unknown, IBS-associated intestinal bacteria escaped the PCR analyses applied in this study.

No significant changes in SCFAs or bacterial enzyme activities in the fecal samples were observed during the intervention. Hence, these results do not support the hypothesis that the modulation of SCFA production is involved in IBS symptom reduction, as postulated by Tana et al. (299). Those authors showed that increased acetic acid and propionic acid levels in the fecal samples of IBS patients were positively correlated with more severe symptoms, impaired quality of life and negative emotions (299).

Based on the results presented in Study III, it is evident that more emphasis should be placed on the investigation of the mechanisms behind probiotic functionality in IBS. Moreover, further research is needed to shed
### Results and Discussion

**Table 5. Fecal bacterial numbers (log_{10} bacteria per gram dry weight of feces) at baseline and during probiotic (n = 22) or placebo (n = 21) supplementation.**

<table>
<thead>
<tr>
<th>Real-time PCR target</th>
<th>Baseline (min-max)</th>
<th>Placebo (min-max)</th>
<th>Baseline-adjusted marginal means during supplementation (3 months - 6 months)</th>
<th>Ratio Probiotic/Placebo</th>
<th>Mean (95% CI)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atobopium group</strong></td>
<td>10.5 (9.61-11.31)</td>
<td>10.2 (7.41-11.71)</td>
<td>10.3 (10.08-10.51)</td>
<td>10.6 (10.33-10.77)</td>
<td>0.6 (0.27-1.13)</td>
<td>0.103</td>
</tr>
<tr>
<td><strong>Bacteroides fragilis</strong></td>
<td>7.5 (4.69-9.90)</td>
<td>6.5 (4.69-10.49)</td>
<td>6.6 (6.08-7.20)</td>
<td>6.9 (6.31-7.46)</td>
<td>0.6 (0.09-3.65)</td>
<td>0.537</td>
</tr>
<tr>
<td><strong>Bacteroides-Prevotella-Porphyromonas</strong></td>
<td>10.7 (8.97-11.60)</td>
<td>10.6 (9.19-11.15)</td>
<td>10.6 (10.44-10.74)</td>
<td>10.5 (10.33-10.64)</td>
<td>1.3 (0.77-2.09)</td>
<td>0.336</td>
</tr>
<tr>
<td><strong>Bifidobacterium spp.</strong></td>
<td>9.6 (7.79-10.69)</td>
<td>9.2 (5.09-11.52)</td>
<td>9.2 (8.82-9.61)</td>
<td>9.9 (9.45-10.26)</td>
<td>0.2 (0.06-0.85)</td>
<td>0.028</td>
</tr>
<tr>
<td><strong>Campylobacter spp.</strong></td>
<td>5.5 (4.61-7.66)</td>
<td>5.4 (4.61-6.65)</td>
<td>5.4 (5.07-5.63)</td>
<td>5.2 (4.86-5.44)</td>
<td>1.6 (0.63-4.07)</td>
<td>0.314</td>
</tr>
<tr>
<td><strong>Clostridium cocoides-Eubacterium rectale group</strong></td>
<td>11.5 (10.62-12.28)</td>
<td>11.4 (11.02-11.90)</td>
<td>11.4 (11.25-11.51)</td>
<td>11.4 (11.24-11.50)</td>
<td>1.0 (0.67-1.59)</td>
<td>0.884</td>
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<tr>
<td><strong>Clostridium perfringens group</strong></td>
<td>5.9 (4.22-8.64)</td>
<td>5.5 (4.22-7.17)</td>
<td>5.8 (5.39-6.15)</td>
<td>6.0 (5.54-6.42)</td>
<td>0.7 (0.17-2.86)</td>
<td>0.599</td>
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<tr>
<td><strong>Desulfovibrio desulfuricans group</strong></td>
<td>6.9 (4.92-8.97)</td>
<td>7.0 (4.92-8.87)</td>
<td>7.0 (6.73-7.31)</td>
<td>7.1 (6.83-7.43)</td>
<td>0.8 (0.30-2.05)</td>
<td>0.605</td>
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<tr>
<td><strong>Enterococcus spp.</strong></td>
<td>7.8 (6.21-8.86)</td>
<td>7.8 (6.68-9.39)</td>
<td>7.7 (7.47-7.96)</td>
<td>7.8 (7.53-8.02)</td>
<td>0.9 (0.40-1.94)</td>
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<td><strong>Echerichia coli subgroup</strong></td>
<td>6.9 (4.80-8.61)</td>
<td>7.7 (4.80-10.56)</td>
<td>6.7 (6.36-7.11)</td>
<td>7.1 (6.70-7.47)</td>
<td>0.5 (0.13-1.59)</td>
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<td><strong>Faecalibacterium prausnitzii</strong></td>
<td>9.1 (7.74-10.04)</td>
<td>9.0 (7.68-9.52)</td>
<td>8.9 (8.69-9.06)</td>
<td>8.8 (8.64-9.03)</td>
<td>1.1 (0.59-2.05)</td>
<td>0.763</td>
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<td><strong>Lactobacillus group</strong></td>
<td>8.2 (6.55-9.36)</td>
<td>7.9 (4.99-9.37)</td>
<td>8.2 (7.89-8.46)</td>
<td>8.0 (7.70-8.27)</td>
<td>1.5 (0.61-3.93)</td>
<td>0.353</td>
</tr>
<tr>
<td><strong>Ruminococcus productus-Clostridium cocoides</strong></td>
<td>6.8 (4.80-8.26)</td>
<td>6.9 (5.79-9.28)</td>
<td>6.9 (6.71-7.11)</td>
<td>6.8 (6.54-6.96)</td>
<td>1.5 (0.74-2.84)</td>
<td>0.267</td>
</tr>
<tr>
<td><strong>Veillonella spp.</strong></td>
<td>8.3 (6.43-9.62)</td>
<td>8.2 (4.92-9.92)</td>
<td>8.1 (7.83-8.38)</td>
<td>7.9 (7.64-8.20)</td>
<td>1.5 (0.62-3.80)</td>
<td>0.345</td>
</tr>
</tbody>
</table>

*anova for repeated measurements, using logarithmically (log_{10}) transformed values and baseline as a covariate. Bold indicates statistically significant value.
more light on the role of intestinal microbiota in the pathogenesis of IBS. This would greatly facilitate the discovery and establishment of microbiota-related markers specific for the disorder.

5.5 SCREENING OF PATHOGENS IN FECAL SAMPLES OF IBS PATIENTS AND HEALTHY CONTROLS (IV)

As discussed in the Section 2.3.5, the predominant enteric bacteria in the fecal samples of IBS subjects have been widely studied with different DNA-based techniques (58, 150, 189, 202, 299). To our knowledge, however, there are relatively few reports focusing on the systematic screening of enteric pathogens, the abundance of which is likely to be significantly lower as compared to the dominant microorganisms present in the gut. Moreover, as PI-IBS is considered a prolonged state of functional gastrointestinal symptom triggered by an acute gastroenteritis, the level of the original causative agent may be much lower or even undetectable after the acute phase of an enteric infection has passed. Thus, much of the data regarding the role of pathogens as causative agents of IBS is likely to be limited, since the routine clinical investigations commonly rely on bacterial culturing techniques possibly lacking the sufficient sensitivity for detecting low levels of pathogens from fecal specimens.

The rationale of the Study IV was to investigate the presence and abundance of selected intestinal pathogens in fecal samples of IBS subjects and healthy controls even at low detectable levels to shed light on the putative role of these bacteria in the pathogenesis of IBS. For this, real-time PCR procedures, which utilized 12 previously designed oligonucleotide primer pairs targeted either to a virulence gene or the 16S rRNA gene for the detection of an extensive set of intestinal pathogens, were optimized for application in our SYBR Green I-based real-time PCR quantification system.

The primer sequences, target organisms, PCR product sizes, annealing temperatures, and references are summarized and listed in Table 4. In the in silico analysis, no cross-reaction with closely related or undesired bacterial species was observed with any of the primer pairs tested. An excellent dynamic range of five logs was obtained with seven out of eight assays when serially diluted chromosomal DNA was used as template. The exception was Bacillus cereus group assay, with the dynamic range of four logs due to the excessive formation of primer-dimers. In addition, no signals above the detection limit from negative controls were obtained with any of the assays.

The fecal samples of 96 IBS and 23 control subjects were analyzed with newly optimized real-time PCR assays targeting the most relevant intestinal pathogenic bacteria. The IBS subjects had originally been recruited for two independent studies and thus the inclusion and exclusion criteria were not identical. Due to two independent sample sets with different classification criteria, the IBS-D and IBS-M subgroups were combined for this study. As a consequence, the IBS group consisted of subjects with IBS-D or IBS-M (81 subjects) and IBS-C (15 subjects). In the analysis of Aeromonas spp., B. cereus group, EHEC and EPEC, L. monocytogenes, Salmonella spp., and Y. enterocolitica, all samples from both subject groups remained below the limit of detection (~10^5 bacterial genomic equivalents per gram of fecal sample) of the respective real-time PCR analyses. Conversely, in the analysis of S. aureus, Cl. perfringens, H. pylori and Campylobacter spp., positive signals were obtained.

The most important finding of Study IV was that a total of 15 IBS samples (prevalence of 17%) tested positive for S. aureus with a thermonuclease (nuc) gene -targeting qPCR
assay, the numbers ranging from $2.5 \times 10^4$ to $4.0 \times 10^7$ genomic equivalents per gram of feces (Figure 11), whereas none of the healthy controls were positive for *S. aureus* ($p < 0.05$; Pearson’s Chi-square test). The true positivity of *S. aureus* findings was confirmed by sequencing the PCR amplicons obtained. All 15 sequences were found to be 100% identical and showed complete accordance with *S. aureus* subsp. *aureus* nuc gene. The *S. aureus*-positive IBS samples were relatively evenly distributed among the two subcategories as the frequencies of positive samples in IBS-D&M and IBS-C subgroups were 16% and 13%, respectively. No clear relation to age or gender was observed among the *S. aureus* positive samples. To our knowledge, *S. aureus* has not been reported to be connected with the onset of IBS, although recent evidence suggests that IBS can be triggered by an episode of acute gastroenteritis, as discussed in Section 2.3.4.

*S. aureus* is a food-borne pathogen known to cause food poisoning but it is difficult to obtain accurate estimates of the precise incidence of *S. aureus* intoxications, since many cases are not specified or reported. The disease occurs as a result of the ingestion of foods or beverages containing one or more preformed enterotoxins (SE) produced by the species and it is characterized by symptoms including diarrhea, nausea, and abdominal cramps (311). Classically, SEs have been divided into five major serological types (SEA, SEB, SEC, SED, and SEE) on the basis of their antigenic properties (291) but relatively recently, nine new types have been discovered (SEG to SEO) (166). In this study, we targeted the thermonuclease gene as a common *S. aureus* marker in the real-time PCR analyses. Therefore, the occurrence of enterotoxin producing strains within the *S. aureus* positive samples detected remains to be evaluated. It is noteworthy, however, that in a study by Pinto and coworkers (240), a total of 40 out of 131 food isolates analyzed were positive for the se genes (prevalence of 31%), the sec genotype being the most frequent.

In the analysis of *Clostridium perfringens* (α-toxin encoding *cpe* gene), sporadic positive signals were detected in the samples of IBS patients but also healthy controls, and hence no statistically significant differences were observed (Figure 11). In addition to the α-toxin gene-targeted real-time PCR, all samples were screened for *cpe*, which is a gene encoding enterotoxin (CPE), a causative agent of *Clostridium perfringens* Type A food poisoning resulting in diarrhea and abdominal cramps (37). No indications of the presence of the *cpe* gene possessed by *Clostridium perfringens* were observed in either of the subject groups. The obtained results along with the fact that *Clostridium perfringens* is a commensal species in the stool of some normal healthy subjects (173) implies that there was no association between *Clostridium perfringens* and the pathogenesis of the IBS at least within the subjects of this study.

The most common enteric pathogens associated with PI-IBS include the foodborne pathogens *C. jejuni*, *E. coli* O157:H7, and *Salmonella* spp. In this study, we found *C. jejuni* in the fecal sample of one IBS patient belonging to the IBS-D&M subgroup, but no signs of *Salmonella* or enteropathogenic and enterohemorrhagic *E. coli* were observed. The association between *H. pylori* infection and IBS patients has been previously reported by Su et al. (290). They discovered by applying 13C urea breath test and histological examination (Gram stain) of tissue obtained at endoscopy that 33 of 69 patients included in their study (47.8%) had *H. pylori* infection, which was associated with functional dyspepsia. Here, the occurrence of *H. pylori* was detected in the samples of three IBS subjects with the average age of 57 years the numbers ranging from $8.3 \times 10^5$ to $1.0 \times 10^7$ genomic equivalents per gram of fecal specimen, while no positive cases were observed among control subjects.
Results and Discussion

Figure 11. Real-time PCR results of the assays for Staphylococcus aureus nuc gene (A) and Clostridium perfringens plc gene (B). The values are target genomes per gram of fecal sample (log_{10} values). The detection limit is set to 10^4 bacterial genomes. The abbreviations: IBS-C and IBS-D&M stand for constipation-predominant irritable bowel syndrome and diarrhea-predominant or mixed-type irritable bowel syndrome, respectively.

It is worth mentioning, however, that the most common habitat of H. pylori is the human stomach and the beginning of the upper digestive tract. In this study, all the analyses were carried out on fecal samples, which may explain the lack of positive cases.

Although previous gastroenteritis was not an inclusion criterion for the IBS subjects in this study, the obtained results in conjunction with earlier findings by other researches suggests that a number of different gastroenteritis-inducing bacteria may be involved in IBS. Hence, it is indeed likely that the inflammatory response of the host rather than a particular individual pathogenic species is probably the key factor triggering the onset of IBS (62). It remains unclear, however, whether the different pathogenic species pose an equal risk of developing the disorder.

We applied the newly optimized panel for the screening of an extensive set of fecal DNA samples of IBS subjects in order to shed light on the putative role of pathogenic bacteria in IBS. Although significant differences in the prevalence of S. aureus between the study groups were observed, its importance in giving rise to IBS symptoms requires further studies. Nevertheless, the results obtained indeed support the earlier suggestions with regards to the role of intestinal pathogens in IBS.
6. CONCLUDING REMARKS

The intestinal microbiota is a complex microbial ecosystem, consisting of 10 times more cells than the human body itself. The primary objectives of the work presented in this thesis were i) to develop and validate real-time PCR-based assays for the analysis of commensal intestinal bacteria and the most common intestinal pathogens; and ii) to assess the potential of this technique to identify putative differences between the microbial community structure of healthy individuals and patients suffering from IBS. To our knowledge, real-time PCR technique has not been applied previously on this scale for analysis of the intestinal microbiota of IBS patients.

The combination of assays developed and applied in this study has an overall coverage of 300-400 known bacterial species, along with the number of yet unknown phyotypes. Therefore, this comprehensive set of assays provides good means for screening the intestinal microbiota and identifying microbes putatively associated with IBS. In addition to research focusing on IBS, the set of PCR assays developed and validated here is also applicable to microbiota screening in other intestinal disorders or conditions.

Real-time PCR was demonstrated to be a superior method for detection and quantification of single bacterial species or larger groups from complex microbial community present in intestinal samples regardless of the evident challenges in the accurate data extrapolation due to the variation in the 16S rRNA gene operon copy numbers within different bacterial species. One of the key benefits of the qPCR technique is its sensitivity. The comprehensively optimized assays enabled the quantification of at least approximately $10^{9}$ bacterial genomes per gram of fecal sample, which corresponds to the sensitivity to detect 0.00001% subpopulations of the total fecal microbiota. This exceeds the sensitivity of any other DNA-based quantification technique available.

Although molecular detection methods have apparent benefits over conventional bacterial culturing, they possess several potential pitfalls that must be carefully taken into account. The main issues for quantification of nucleic acids in complex microbial communities are DNA extraction from different sources or sample types with constant efficiency and sufficient purity, as well as critical selection of the primers or probes to be applied. If the isolated DNA does not correctly represent the microbial composition of the original sample due to inefficient cell disruption or inadequate DNA purification procedure, the subsequent molecular analysis will be biased and thus will not serve its purpose.

Moreover, inadequate primer selection will further hamper interpretation of the results due to potential cross-reactions with unwanted DNA or mismatches preventing the amplification of the target organisms. In designing real-time PCR assays, researchers rely solely on the sequence data deposited in the public or private databases. Therefore, any oligonucleotides used as probes or primers should be considered specific only in relation to the current sequence data available. Fortunately, the available sequence information on intestinal bacterial species is continuously increasing, facilitating the design of more specific real-time PCR assays and also enabling in silico re-evaluation of the primers already in routine use.

The intestinal microbiota alterations discovered here and also by others support the hypothesis that microbes are likely to contribute to the pathophysiology of IBS. However, the central question is whether the microbiota changes described represent the
cause for, rather than the effect of, disturbed gut physiology. Therefore, more studies are needed to determine the role and importance of individual microbial species or groups in IBS. Current knowledge of the bacterial species or, more specifically, the specific genes associated with different GI symptoms is very limited, which hampers the understanding of functional consequences of specific alterations in microbiota composition.

In addition, further work regarding the cause-effect relationship between specific members of intestinal microbiota and microbial metabolites is warranted. The recent findings indeed imply that elevated levels of certain SCFAs may play a role in the onset and maintenance of IBS symptoms. Thus, more focused research is required to shed light on whether the accumulation or types of SCFAs and metabolic cross-feeding between different bacteria are able to contribute to IBS.

The heterogeneous nature of IBS makes it a difficult condition to study. For example, the variation in the intestinal microbiota composition between the subtypes hampers the extrapolation of microbial characteristics from one IBS subtype to another. Furthermore, the independent surveys thus far have been carried out with a relatively small number of study subjects with varying recruitment criteria and the findings of different studies have been obtained by applying diverse sample processing and analysis techniques. The abovementioned differences obstruct reliable comparisons of independent studies and may explain the many contradictions between the results published. In future studies, it is essential to confirm the microbial alterations discovered in this study and elsewhere by using a larger set of IBS samples of different subtypes, preferably from various geographical locations, and by applying carefully validated and controlled study protocols.
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Teemu Rinttilä
8. REFERENCES


References


References

References


References


References


67

References

References


References


References