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Title: 16S rRNA gene profiling: Direct and indirect applications for clinical microbiology
Issue Date: 2020-12-08

CHAPTER 1

General introduction, aim and outline of the thesis

THE HUMAN MICROBIOTA AND MICROBIOME

The human body is colonised by a diversity of microorganisms, including archaea, bacteria, bacteriophages, fungi, protozoa and viruses. This is collectively known as the human microbiota. The traditional estimate is that these 10-100 trillion microorganisms outnumbers the human cells by at least a factor of 10 (1). More recent estimates have lowered that ratio to 3:1 or even parity (2). Together, these microorganisms possess approximately 3 million unique genes; 150 times more genes than the human genome (3). The microorganisms and their collection of genes are called the human microbiome or our second genome, which serves as a functional expansion of the human genome. The genes that are added in this way to our own collection encode various types of enzymes that play a critical role in important physiological processes, such as metabolism and immunity. Furthermore, the microbiota prevents colonization and/or outgrowth of pathogens, also known as colonisation resistance, preventing infectious diseases. Bacteria are the best studied group of microorganisms in this context, as they overwhelmingly outnumber the other microorganisms present in the human microbiota by an orders of magnitude of 2-3 (4, 5). Based on their similarities and relationships, bacteria can be arranged into taxonomic groups or taxa (**Figure 1**).

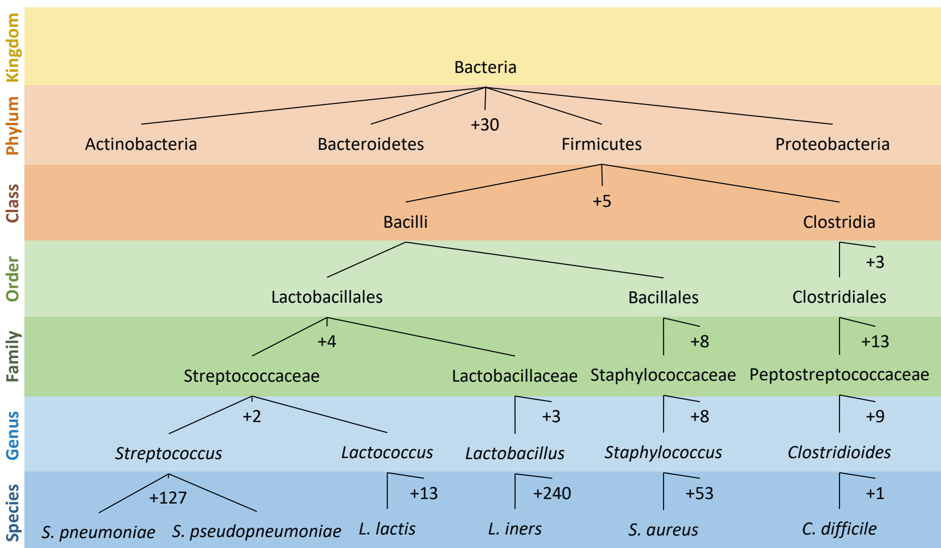

Figure 1. Bacterial taxonomy with bacterial species present in this thesis as examples. In the bacterial kingdom, bacteria are arranged in small but homogenous groups or taxa. Species is the basic taxonomic group. Groups of species are collected into genera. Groups of genera are collected into families, families into orders, orders into classes, and classes into phyla, the major lineages of the bacterial kingdom. The number of taxonomic groups are based on the List of Prokaryotic Names with Standing in Nomenclature (6).

Acquisition of the human microbiota is believed to be initiated at birth. Recent reports of bacteria present in the placenta, fetal membranes, amniotic fluid, and umbilical cord blood of healthy term pregnancies have challenged this belief (7, 8). However, the '*in utero* colonization hypothesis' remains the subject of debate. The critical issue concerns the low microbial biomass of these body sites and the potential bias from background, contaminant DNA, which increases the risk of false positive results (9). Regardless of intrauterine exposure, the microbiota of the neonate is influenced by mode of delivery with the microbiota sourced from the mother's vagina during delivery (predominately *Lactobacillus* and *Prevotella* spp.) or from skin with caesarean section (predominantly *Staphylococcus* and *Corynebacterium* spp.) (10, 11). After birth, the microbiota undergoes significant reorganization driven by body site (12). Type of feeding is another important driver of microbiota maturation (8, 13). Approximately three years postpartum, the microbiota composition becomes more stable and roughly resembles that of adults, consisting predominantly of bacteria within the phyla Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria (14). At that time, each body site contains a 'core' as well as 'variable' set of bacteria. The microbiota composition of each body site is broadly similar among humans worldwide due to the shared physical and chemical features (15). Differences in microbiota composition of a specific body site between humans originate from environmental factors that may vary significantly among humans, such as hygiene, lifestyle, geographic location, medication and diet (16, 17). Accordingly, the microbiota of a specific body site is closely related to humans sharing a home (18, 19).

During the course of our life, the microbiota composition of each body site varies in terms of membership (what is present) and structure (quantity of a member compared to the total). These changes are mainly caused by environmental factors, such as physical interaction between individuals, antibiotic exposure, changes in both hygiene and lifestyle, but also by age (20, 21). The degree of variation depends on the complexity of the microbiota as a diverse microbiota tends to be less stable compared to a less diverse microbiota (22, 23). Furthermore, the degree of variation is also a personal feature, meaning that the microbiota of a specific body site can vary more in one individual compared to another (24, 25).

THE HUMAN MICROBIOTA AND ASSOCIATIONS WITH DISEASE

In 2006, Gill and colleagues were the first to reveal that the microbiota of the human gastrointestinal tract encodes for more enzymes than the human genome itself (26). This finding highlighted the crucial role of the human gut microbiota in health and laid the groundwork for further research to investigate the association between the human gut microbiota and disease. The best evidence to highlight the importance of the human gut microbiota in health and disease was obtained from clinic studies treating patients with antibiotics for *Clostridioides difficile* infections (27). *C. difficile* is a spore-forming bacterium and

asymptomatic carriage in the normal population is estimated to be 3-15%. The healthy gut microbiota, comprising a wide range of bacteria predominantly within the phyla Firmicutes and Bacteroidetes, inhibits growth of *C. difficile*. This defence system includes competition for nutrients, production of antimicrobial compounds, and regulation of secondary bile acid metabolism, which in contrast with primary bile acids, inhibit *C. difficile* spore germination (28-30). Antibiotic treatment changes the composition of the gut microbiota, reducing its diversity and leading to a decreased inhibition of the growth of *C. difficile*. An overgrowth of *C. difficile* is the most important cause of hospital-acquired diarrhoea and is usually treated with another round of antibiotics (31). In approximately 80% of *C. difficile* infected patients, the infection is cleared after treatment. However, in almost 20% of the patients, antibiotic treatment leads to further disruption of the gut microbiota, reduced colonization resistance and subsequent recurrence of the *C. difficile* infection. After repeated use of broad-spectrum antibiotics to treat the recurrent infection, the microbiota is completely out of balance, which is called dysbiosis (**Figure 2**). In early 2011, a faecal microbiota transplant (FMT) obtained from a healthy donor restored the healthy gut microbiota and prevented recurrent episodes of diarrhoea in approximately 94% of the patients (32). This study demonstrated a strong association between the gut microbiota composition and *C. difficile* infection related disease. Furthermore, this finding remains the best proof-of-principle that the healthy gut microbiota can reproducibly correct severe and specific dysbiosis. To date, FMT remains the primary therapy for patients with recurrent *C. difficile* infections where appropriate antibiotic treatments failed (33-35).

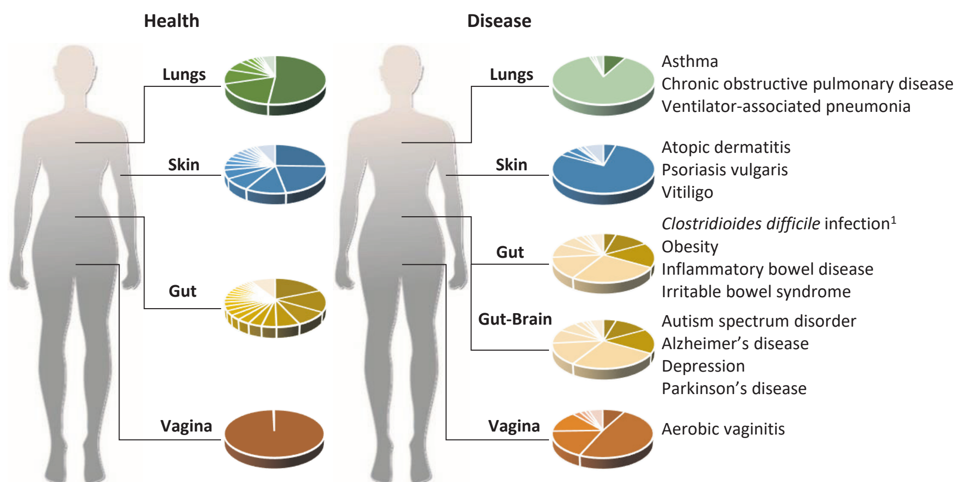

Figure 2. Examples of the Human microbiota in health and disease. Each body site, such as the gastrointestinal tract, respiratory tract, skin and urogenital system, contain a unique composition of microorganisms, called the microbiota. The microbiota composition can change in time (lose or obtain microbial diversity), which might be associated with specific diseases. [1]Microbiota of the gut after antibiotic treatment for *Clostridioides difficile* infection.

In the meantime, a tremendous amount of circumstantial evidence has been collected to suggest a crucial role for the human microbiota in health and disease with a strong focus on the gastrointestinal tract (**Table 1a-d**). Only a few of these human studies have been complemented by mechanistic studies demonstrating causality. For the gut, a hypothesis has been proposed to explore whether dysbiosis is a cause or consequence of a disease (36). According to this hypothesis, (i) each healthy person is genetically susceptible to one or more polygenetic disorders, (ii) environmental factors trigger gut microbial dysbiosis, intestinal inflammation and/or increase gut permeability known as 'leaky gut', (iii) combination of genetic susceptibility and environmental exposure results in polygenetic disorder, and (iv) transplantation of the disease-associated gut microbiota to a genetic susceptible host reproduces the distinct disease phenotype. In the following sections, we will briefly discuss the role of the human microbiota in obesity, inflammatory bowel disease and neuro-psychiatric diseases to show that microbial dysbiosis can have local and systemic effects. Finally, we will highlight the major clinical findings of the respiratory tract microbiota to show that microbiota development early in life might have incredible consequences for future health.

Table 1. Evidence of human studies suggesting a crucial role for microbiota of (**a**) the respiratory tract, (**b**) the gastrointestinal tract, (**c**) the skin or (**d**) the urogenital system in health and disease

a. Microbiota of the respiratory tract

Disorder category	Specific disorder	Evidence
Infectious diseases	Acute respiratory infections	• Early colonization with *Haemophilus influenzae* and *Streptococcus pneumoniae* associated with higher prevalence (13, 105)
	Ventilator-associated pneumonia	• Increase in the order Pseudomonadales (106, 107)
Immune-related/ autoimmune diseases	Chronic obstructive pulmonary disease	• Increased abundance of the phylum Proteobacteria or Firmicutes (108)
	Recurrent wheezing and asthma	• Increased abundance of *Haemophilus influenzae*, *Moraxella catarrhalis* or *Streptococcus pneumoniae* (109-116)
Chronic suppurative lung diseases	Cystic fibrosis	• Increased abundance of potential pathogens (117-119)
	Bronchiectasis	• Increased abundance of potential pathogens (120)
Chronic inflammatory diseases	Chronic rhinitis	• Increased abundance of *Staphylococcus* and *Propionibacteria* spp. (121)
	Chronic rhinosinusitis	• Increased abundance of potential pathogens (122-124)
	Otitis media	• Increased abundance of potential pathogens (125, 126)

b. Microbiota of the gastrointestinal tract

Disorder category	Specific disorder	Evidence
Infectious diseases	*Clostridioides difficile* infections	• Decreased microbial diversity (27, 37-40) • Secondary bile acid metabolism regulated by microbiota inhibits spore germination (30) • 94% of patients cured after faecal microbiota transplant (32)
Metabolic disorders	Obesity	• Increased abundance of short-chain fatty acids producing Firmicutes and decreases abundance of phylum Bacteroidetes (41, 42) • Improved insulin sensitivity after faecal microbiota transplantation, but no effect on weight (43-45)
	Type-2 diabetes	• Decrease in short-chain fatty acids producing bacteria (46-50)
Immune-related/ autoimmune diseases	Celiac disease	• Variable dysbiosis (51-54) • Gluten-free diet restores partly microbiota (55-57)
	Inflammatory bowel disease	• Disturbance of microbial balance (58-61) • Varying response to faecal microbiota transplantation (62-69)
	Irritable bowel syndrome	• Variable dysbiosis (70-72) • Significant decrease in severity score at three months post-faecal microbiota transplant in 65% of the patients (73, 74)
	Multiple sclerosis	• Increased microbiota diversity (75, 76)
	Systemic lupus erythematosus	• Decreased microbiota diversity (77)
	Type-1 diabetes	• Disturbed ratios of the phyla Bacteroidetes and Firmicutes (78-83)
	Rheumatoid arthritis	• Increased *Prevotella* spp. (84, 85)
Neuro-psychiatric diseases	Autism spectrum disorder	• Increased microbial diversity (86-88) • Behavioural symptoms and microbiota improved significantly after faecal microbiota transplant (89)
	Alzheimer's disease	• Possible connection between gut microbiota-synthesized amyloids, lipopolysaccharides, γ-aminobutric acid and the increased permeability of the gut barrier and blood brain barrier with age (90, 91)
	Depression	• Depletion of *Faecalibacterium* and *Coprococcus* spp. (92-95)
	Parkinson's disease	• Increased abundance of short-chain fatty acids producing *Blautia* and *Coprococcus* spp. (96) • Gut bacteria interfere with treatment (97)
Cancer	Colorectal cancer	• Increased abundance of *Bacteroidetes fragilis* (98, 99) • *Fusobacterium nucleatum* promotes chemoresistance to colorectal cancer (100)
Liver diseases	Hepatic encephalopathy	• Increased abundance of the families *Alcaligenaceae* and *Pyrphyromonadaceae* (101) • Faecal microbiota transplant may be potentially effective in preventing long-term recurrence of hepatic encephalopathy (102-104)

c. Microbiota of the skin

Disorder category	Specific disorder	Evidence
Immune-related / autoimmune diseases	Atopic dermatitis	• Increased abundance of *Staphylococcus aureus* (127) • Reduced microbial diversity (128, 129) • Autologous bacterial transplant of coagulase-negative *Staphylococcus* strains reduced *S. aureus* colonization (130) • Allogeneic bacterial transplant of *Roseomonas mucosa* reduced symptoms and *S. aureus* colonization (131)
	Acne vulgaris	• Increased diversity within follicles (132) • Specific *Propionibacteria acnes* strains associated with acne (133)
	Chronic wounds	• Increased abundance of polymicrobial biofilms (134, 135)
	Psoriasis	• Decreased microbial diversity (136-140)
	Rosacea	• Disturbance of microbial balance (141)
	Seborrheic dermatitis	• Imbalance between bacteria and fungi on the scalp surface (142)
	Vitiligo	• Decreased microbial diversity (143)

d. Microbiota of the urogenital system

Disorder category	Specific disorder	Evidence
Polymicrobial syndromes	Bacterial vaginosis	• Shift from *Lactobacillus* spp. dominated vaginal microbiota to a more diverse microbiota dominated by anaerobes (144-147) • Long-lasting improvements in four of five patients with recurrent bacterial vaginosis after 1-3 vaginal microbiota transplant sessions (148)
	Aerobic vaginitis/ desquamative inflam-matory vaginitis	• Shift from *Lactobacillus* spp. dominated vaginal microbiota to a more diverse microbiota dominated by aerobes (149)

Obesity

Obesity is a global health hazard affecting more than 650 million people worldwide and is associated with a higher risk of developing cardiovascular disease, type II diabetes, and liver abnormalities. An imbalance in the energy intake and energy expenditure is considered as the major cause of this condition. Although lifestyle and genetic factors are also considered as the influential determinants of obesity, recent research suggested microbiota to be a key environmental factor that influences obesity. In obese patients, a significant increase in Firmicutes that produce short-chain fatty acids (SCFA) and a decrease in the relative abundance of Bacteroidetes was observed (150). Such altered microbiota composition is believed to result in the upregulation of energy and calories production from the undigested materials, altering the energetic homeostasis. A causal role for the gut microbiota in obesity is strongly supported by mouse models, suggesting that FMT might improve metabolism

(151). Nevertheless, no effect on weight was observed when FMT from lean donors was transferred into obese patients although their insulin sensitivity improved significantly (43-45). So, to date there is no significant proof that FMT is sufficient to induce weight loss, but these studies indicate that the microbiota may be a potential target for therapy.

Inflammatory bowel disease

Other disorders that are rising at an alarming rate worldwide include Crohn's disease and ulcerative colitis. Both are inflammatory bowel diseases affecting parts of the gastrointestinal tract. The chronic inflammation is driven by both genetic susceptibility and environmental factors, such as diet and antibiotic use. Furthermore, dysbiosis of the gut microbiota in patients with Crohn's disease or ulcerative colitis exist as demonstrated by a reduced microbiota diversity and expansion of potentially pathogens (152-154). The decrease in gut microbial diversity is characterised by the depletion of SCFA-producing bacteria, which drive the expansion of regulatory T-cell populations that suppress the inflammatory response in the gut (155). Decreased production of SCFA might result in an increased inflammatory response, leading to chronic inflammation of the gut. Randomised clinical trials with FMT showed promising results for a small subset of patients with ulcerative colitis (62-65, 156). For Crohn's disease, only small, uncontrolled cohort studies have been performed with mixed results (66-69). Since the observed effects have been very modest, FMT should still considered an experimental approach in inflammatory bowel diseases. Currently, 85 clinical trials focussing on FMT and inflammatory bowel diseases have been registered in the ClinicalTrials.gov database.

Neuro-psychiatric diseases via the gut-brain axis

Previous examples showed a relatively strong and local association between microbiota and disease. However, it has been suggested that the gut microbiota can communicate via the neural, endocrine and immune system with the central nervous system. This bidirectional communication system is more commonly referred to as the gut-brain axis (157). Emotional factors, such as stress or depression, influence indirectly the composition of the gut microbiota and neuro-psychiatric disorders frequently coexist with common gastrointestinal diseases associated with gut dysbiosis. Accordingly, it is not surprising that several neuro-psychiatric diseases have been associated with gut dysbiosis, such as autism spectrum disease, Alzheimer's disease, depression, and Parkinson's disease. The strongest results showing that a person's gut microbiota can influence their mental health comes from a recent publication (92), which reported that specific bacteria were reduced in the gut microbiota of patients with depression. Furthermore, they observed a positive correlation between quality of life and the potential ability of the gut microbiota to synthesize a breakdown product of the neurotransmitter dopamine, called 3,4-dihydroxyphenylacetic acid.

Respiratory tract microbiota

Altered microbiota composition has been directly linked to disease, but microbiota development in early life might also have consequences for future health. Microbiota composition of the upper respiratory tract at six weeks of life is strongly associated with both microbial (in)stability as well as with the prevalence of respiratory tract infections in the first two years of life (105). Furthermore, specific microbiota profiles are associated with an increased risk of recurrent wheeze and asthma in later childhood, suggesting long-term effects on host response to environmental triggers (158). The major drivers of microbiota development are type of feeding, crowding, exposure to antibiotic, and last but not least mode of delivery (11, 159). Applying vaginal microbiota transplants to the skin of Caesarean-born children restored their microbiota, resulting one month postpartum in similar skin and oral microbiota as babies born vaginally. However, long-term effects remain to be determined.

MICROBIOTA RESEARCH

Historically, the first microbiota studies started with Sergei Winogradsky in 1885, who investigated the microorganisms in connection with each other and discovered the nitrogen cycle. By mimicking natural soil conditions, he discovered the interconnectedness of microorganisms, that occupy the niches created by their neighbours' activities and use the products of one metabolic pathway as substrates for another. Modern microbiota research started with Venter and colleagues in 2004, who were the first to apply DNA sequencing-based methods on a large scale to study microorganisms within environmental samples (160). Their research revealed the presence of at least 1,800 different species in water samples obtained from the Saragasso Sea, while only a small number of species was expected due to the low nutrient levels of the sea. This pioneering research illustrated that DNA sequencing-based methods, which were not limited to microorganisms that could be cultivated effectively, generate more comprehensive characterisation of microbial communities. In 2008, the Human Microbiome Project was introduced by the National Institutes of Health (15). The project allowed researchers to explore how the human microbiota interacts with the human body in much greater detail than ever before. At that time, high-throughput sequencing platforms were available for comprehensive characterisation of microbial communities, enabling easier detection of a theoretically unlimited number of microorganisms using a culture independent approach. The development of the high-throughput sequencing platforms also led to boosted microbiota research.

Currently, multiple high-throughput sequencing platforms are available including the Illumina, the Pacific Biosciences (PacBio) and the Oxford Nanopore platforms (**Table 2**). The Illumina platforms are presently dominating the market due to its' cost-effectiveness,

Table 2. Technology, platforms and features of the available high-throughput sequencing methods (164, 166-172)

Sequencing technology	Platform	Maximum read length	Throughput	Runtime	Limits
Pyrosequencing	Roche 454 GS Junior series	1000 bp	70 Mb	18 h	High error rate
	Roche 454 GS FLX series	1000 bp	700 Mb	23 h	
Sequencing by synthesis	Illumina iSeq 100 system	2x 150 bp	1.2 Gb	18 h	High DNA concentrations are required
	Illumina MiniSeq system	2x 150 bp	7.5 Gb	24 h	
	Illumina MiSeq series	2x 300 bp	15 Gb	55 h	
	Illumina NextSeq series	2x 150 bp	120 Gb	30 h	
	Illumina HiSeq 4000 system	2x 150 bp	1500 Gb	3.5 days	
	Illumina HiSeq X series	2x 150 bp	1800 Gb	< 3 days	
	Illumina NovaSeq 6000 system	2x 250 bp	6000 Gb	38 h	
Sequencing by ligation	Thermo Fisher Scientific SOLiD 5500 series	2x 50 bp	320 GB	10 days	Reported problems in sequencing and relatively slow compared to other methods
	MGI MGISEQ-200 system	2x 100 bp	60 GB	48 h	
	MGI BGISEQ-50 system	50 bp	225 GB	< 15 h	
	MGI BGISEQ-500 system	2x 100 bp	520 GB	< 9 days	
	MGI MGISEQ-2000 system	400 bp	1440 GB	38 h	
	MGI MGISEQ T7 system	2x 150 bp	6 TB	< 24 h	
Semiconductor sequencing	Ion Torrent PGM series	400 bp	1-2 Gb	7.3 h	High error rate
	Ion Torrent Proton system	200 bp	15 Gb	2.5 h	
	Ion Torrent GeneStudio S5 series	600 bp	1.5-4.5 Gb	7 h	
Single-molecule, real-time	Pacific BioSciences PacBio RSII	~ 20 Kb	0.5-1 Gb	4 h	Very expensive equipment and/or high error rate
	Pacific BioSciences PacBio Sequel	10-60 Kb	3-8 Gb	6 h	
	Oxford Nanopore Flongle	~ 2 Mb	2 Gb	<16 h	
	Oxford Nanopore MinIon	~ 2 Mb	50 Gb	< 48 h	
	Oxford Nanopore GridIon	~ 2 Mb	250 Gb	< 48 h	
	Oxford Nanopore PromethIon	~ 2 Mb	5.2 Tb	< 72 h	

high-quality data, and relative long read length (161). Illumina follows the principle of sequencing by synthesis technology, which includes a DNA polymerase and reversible chain terminator nucleotides for all four bases represented by a different fluorescent dye (162). Sequencing involves the ligation of specific adaptors to both ends of short DNA fragments, which will subsequently hybridize with specific oligonucleotides on a microfluid flow cell. The labelled nucleotides are then introduced and incorporated into the growing complementary strand by the DNA polymerase. Sequential images are captured and

analysed to identify the nucleotide that is incorporated in each synthesis cycle, leading eventually to the complete sequence of the DNA fragment or read. The PacBio platforms are also based on the sequencing by this principle. However, instead of making an image after each synthesis cycle, the signals emitted upon corporation of the nucleotides are detected in real-time (163). This allows generation of longer reads but also results in much higher error rates, which can be addressed by increasing the sequencing depth (i.e. generating more sequences per specimen) (164). Another advantage of the PacBio platforms is that a single DNA fragment is sequenced instead of amplifying the DNA fragment before sequencing, reducing amplification bias. Like the PacBio platforms, the Oxford Nanopore platform is a single-molecule real-time sequencing platform, but the technology is completely different. Instead of binding DNA fragment onto a solid surface for sequencing, Oxford Nanopore sequencing technology is based on protein pores within a conductive electrolytic solution which creates a small potential gradient across these nanopores (165). The ionic current is modulated when a DNA fragment traverses through a nanopore and each of the four bases results in a different signal that can be detected in real-time, making it a very fast technology.

The advancements in high-throughput sequencing technologies provides the opportunity to choose the most appropriate sequencing platform to address a specific scientific question. For example, the Illumina Miseq platform is commonly used for 16S ribosomal RNA (rRNA) gene profiling, whereas the Illumina HiSeq platform, providing higher throughput, is more suitable for whole metagenome shotgun sequencing (164). The third-generation sequencing platforms of PacBio and Oxford Nanopore are more appropriate for addressing scientific question requiring longer reads, such as whole genome shotgun sequencing (164). The different methods based on high-throughput sequencing technology are outlined in the next sections.

16S rRNA gene profiling

The most widely used method for microbiota analysis is 16S rRNA gene profiling or, more specific, amplicon sequencing of the 16S rRNA gene. This method consists of five steps, starting with DNA extraction (**Figure 3**). In order to achieve effective DNA extraction, several procedures have been developed, including the chemical or mechanical disruption of cells, lysis using detergents, or a combination of these approaches. The choice of the most optimal DNA extraction method is greatly dependent on the specimen type and target bacteria to be investigated, since some cell types may resist common mechanical or chemical lysis methods (173, 174).

The second step is the amplification of a DNA fragment that is present in all bacterial genomes, is copied from generation to generation with a high degree of precision, mutates very slowly, and demonstrates considerable sequence diversity among different bacteria. The three rRNA genes encoding for the 16S, 5S and the 23S RNA components of the ribosome and the internal transcribed spacer sequences separating these genes fulfil

these requirements. Of these genes, the 16S rRNA gene is the most widely used genetic marker ever since Carl Woese selected it for studying microbial similarity (phylogenetic relationships) (175-177). The 16S rRNA gene has a length of approximately 1540 nucleotides and contains nine hypervariable regions: V1 to V9. These hypervariable regions exhibit variable degrees of sequence diversity among different bacterial genera. The V1-V2, V3-V4 or V4 is most often targeted in microbiota studies. None of the regions allows differentiation of all bacteria at species level.

Figure 3. Flowchart of 16S rRNA gene profiling for microbiota analysis. DNA is extracted from all cells in a clinical specimen and subjected to a PCR targeting one or two regions of the 16S rRNA gene. All obtained amplicons are sequenced using a high-throughput sequencing platform. Subsequently, the generated reads are classified using a bioinformatics pipeline combined with a reference database. Data analysis of the classified sequences results not only in identification of all members of the bacterial community but also reveals its compositional structure.

The third step consists of library preparation and the sequence reaction itself. As mentioned before, most often the Illumina technology is used because of its' cost-effectiveness and high-quality data. Some researchers use third generation sequencing platforms to be able to sequence the whole 16S rRNA gene (178). However, it is important to note that even sequencing of the whole 16S rRNA gene may lack the discriminatory power to classify bacteria down to species level (179). For example, the sequence similarity of the whole 16S rRNA gene of the *Streptococcus* species within the *Streptococcus* mitis group is so high (≥97%) that these species cannot be differentiated based on the 16S rRNA gene.

The fourth step is classification of the generated reads using a bioinformatics pipeline combined with a reference database. Most pipelines are based on the assignment of reads to operational taxonomic unit (OTU), meaning that reads are clustered based on their degree of similarity. Some pipelines, such as QIIME, compute the similarity between a pair of reads as the percentage of nucleotides that agree in a pairwise sequence alignment.

Typically, a similarity threshold of 97% is used, which was derived from an empirical study that showed most strains had 97% 16S rRNA sequence similarity (180). Other pipelines, such as MOTHUR, the Ribosomal Database Project II (RDP-II) Classifier and the Illumina Miseq software, use shorter DNA fragments (k-mers) instead of the whole sequence to assign reads to OTUs (181). Both types of pipeline select a single read from each OTU as a representative sequence after assigning reads to OTUs. The representative sequence is taxonomically annotated, and all reads within the OTU inherit that same annotation. More recent developed pipelines, such as QIIME 2 (182) and NG-TAX 2.0 (183, 184), use amplicon sequence variants (ASVs) instead of OTUs. ASVs are exact match sequence clusters, which can be separated from error-reads that are assumed to be present at a relatively low abundance. Filtering out sequencing errors improves taxonomic identification. Accurate taxonomic identification of the generated reads also depends on the content of the employed reference database, such as the SILVA, Greengenes or RDP-II reference database (185). These reference databases differ in quality of the reference sequences, the completeness and reliability of the corresponding annotations, and the taxonomic diversity covered by the reference databases due to differences in sources, quality criteria and taxonomy curation methods (**Table 3**). Furthermore, the update status of the reference databases influences their contents since names of organisms as well as taxonomic paths change quickly these days, and the speed by which novel bacterial species, genera and even families are discovered has vastly increased, requiring continuous updating of the reference databases. Both the use of different pipelines and reference databases can result in significant differences between taxonomic classifications (186). Currently, the most commonly used pipeline is QIIME, because of its user-friendliness and the analysis possibilities. The GreenGenes database is still the gold standard for taxonomic classification, but the preference is shifting towards the SILVA database, which has the richest taxonomy of the available databases and is continuously updated.

Finally, the sequencing data can be analysed. The collection of obtained sequences is representative of the bacterial community as a whole in terms of membership (i.e. what is present), and relative abundances, (how many 16S rRNA genes of a member is present compared to the total). Absolute quantification is impossible because a variable number of copies of the rRNA genes are present in each genome depending on the bacterial species (191, 192). Furthermore, the microbiota composition can also be described in terms of alpha and beta diversity (181). Alpha diversity is a measure of taxonomic diversity within a specimen and is expressed with the Shannon index. A high Shannon diversity refers to a specimen with a diverse spectrum of bacteria. In contrast to alpha diversity, beta diversity is a measure of taxonomic diversity between specimens. Similarity between specimens is represented by the distance between specimens across the 3-principal coordinates (PC1, PC2 and PC3). A larger distance between two specimens indicates a large difference in microbiota composition between the two specimens.

Table 3. Features of the available 16S rRNA gene reference databases

Reference database	SILVA Ref NR99 (187)	GreenGenes (188)	NCBI (189)	RDP-II (190)
Sequence source	EMBL-Bank	Multiple sources, but mainly GenBank	GenBank	INSDC databases
Quality criteria	• ≥1,200 bases • <2% ambiguous nucleotides • <2% homopolymers • <2% vector contamination • Confirmed rRNA sequences • No 99% identical sequences	• >1,250 bases • Confirmed rRNA sequences • No 99% identical sequences	• Validation and QA evaluation check for data conflicts and data completeness • Details are not published	• Confirmed rRNA sequences • Details are not published
Taxonomy curation	Phylogenetic tree-guided manual curation approach	Phylogenetic tree-guided manual curation approach	Manual review	RDP Classifier
Lowest taxonomic group	Genus	Species	Species	Genus
Last released version				
• Version number	SSU 138 Ref NR99	gg_13_5_99	Release 95	Version 16
• Release date	December 2019	May 2013	July 2019	February 2016
• Total nr of sequences	510,984	203,452	27,212,750	3,356,809
• Nr of bacterial sequences	431,785	203,452	22,769	3,356,809
Update status	Regularly updated	Not expected	Unknown	Unknown
Remarks	The latest version is free for commercial and other non-academic users	The current gold standard	Not applicable	Not applicable

EMBL: European molecular biology laboratory; INSDC: international nucleotide sequence database collaboration; NCBI: national center for biotechnology information; RDP: ribosomal database project.

It is important to note that incorporating technical controls is necessary to derive reliable conclusions (193). Positive controls ensure that all procedures are correctly performed and that none of the steps have introduced false negative results. Negative controls allow the control of background-contaminating DNA derived from the environment, reagents and/or consumables used during sample processing. Incorporation of negative controls is particularly relevant for studies with low microbial biomass specimens, since even low amounts of background-contamination could have an impact (9). Especially in these studies the connection between the specimens and the corresponding negative controls needs to be carefully evaluated to avoid heated discussions (194).

Whole metagenome shotgun sequencing

16S rRNA gene profiling results only in a comprehensive overview of the bacterial genera present in a specimen. To obtain more information, whole metagenome shotgun (WMS) sequencing can be performed which includes sequencing of all the DNA present in a specimen instead of only a specific DNA fragment (195). To achieve this, the extracted DNA of a specimen is not amplified by PCR, but randomly cut into smaller fragments before sequencing. The generated reads are assembled using specialised software. Based on the obtained consensus sequences, the different bacteria as well as other microorganisms, such as fungi, protozoa and DNA viruses, can be identified down to species level. Another advantage of WMS sequencing is that it produces relative abundance information for all genes present in a specimen, which gives insight in the function of the present microorganisms (195). In general, the determination of functional gene composition involves two steps with various bioinformatics pipelines. The first step is gene prediction, which includes identification of sequences that may (partially) encode proteins. The second step is gene annotation, which includes comparison of the identified protein encoding sequences with a database of protein sequences annotated with their matching function. These data can be used to obtain insight into the antibiotic resistance and virulence profile of the microbiota, but also into the metabolic diversity of the microbiota. Furthermore, this analysis of genomic DNA (genomics) together with the analysis of gene expression (transcriptomics), protein composition, structure, and activity (proteomics) and chemical processes involving metabolites (metabolomics) are important tools to understand the relation between the human microbiota and disease (196).

Important to note is that during WMS sequencing also human DNA present in a specimen is sequenced. The proportion of human DNA differs significantly by body site and specimen collection method. For example, stool specimens comprise less than 10% of human DNA, while other specimens such as saliva, throat and vaginal swabs comprise more than 90% of human DNA (15, 197). A high proportion of human DNA means that only a limited fraction of the generated reads represents the microbial community. Recently, Pereira-Marques and colleagues showed that high proportions of human DNA reduce the sensitivity of WMS sequencing for characterisation of the microbiome, in particular to detect low abundant bacterial species (198). This study highlights the importance of careful design of WMS sequencing experiments to maximize microbiome analysis. The high number of human sequences are also subject of an ethical discussion. Although human DNA is filtered from the dataset, the discussion remains how to protect patient privacy. It may be plausible that the human DNA sequences obtained with WMS sequencing could be used to screen against a panel of known disease-causing genetic variants for example breast cancer. Providing patients with information regarding a potential genetic disease via such an assay is an ethical concern (199).

Both the technical challenge and the ethical issue of WMS sequencing associated with human DNA, but also the higher costs and more complex data analysis are reasons why WMS sequencing has been implemented less than 16S rRNA gene profiling.

Whole genome shotgun sequencing

Another method that is based on high-throughput sequencing is whole genome shotgun (WGS) sequencing, which is the analysis of the complete DNA sequence of a single microorganism. Bacterial species consist of multiple comparable strains, each containing their own unique DNA sequences which might result in different characteristics. The characteristics of each strain can be determined using WGS sequencing (200). The methodology resembles that of WMS sequencing, except that total DNA of a cultured microorganism is used as input material instead of total DNA extracted from a specimen. After sequencing and subsequent assembly of the generated reads, the genome of the microorganism can be analysed. Multiple approaches can be used to identify the bacterial strain, such as core genome and whole-genome multilocus sequence typing (MLST) (200). Core genome MLST uses the sequence difference in the housekeeping genes (the core genome) to identify effectively bacterial strains. Whole-genome MLST is often used as an extension of core genome MLST. It uses the core genome and all accessory genes for the analysis. These genomic analyses can also be used to determine the virulence and antibiotic resistance profile of the bacterial strain. However, the biggest advantage of WGS sequencing is that the genetic relationships between isolates can be investigated (201-204).

CLINICAL MICROBIOLOGY

As described previously, microbiota research explores how the human microbiota interacts with the human body. The clinical microbiology on the other hand is focussed on the prevention, diagnosis and treatment of infectious diseases. Currently, culture-based methods dominate the routine clinical microbiology, but are gradually replaced by PCR-based methods. Culture-based methods are optimized for the efficient cultivation of known pathogens, meaning that microorganisms that rarely cause disease are missed. Furthermore, culture is hindered due to competition during selective culture and the existence of non- or poorly-cultivable pathogens, such as atypical bacteria causing pneumonia (e.g. *Mycoplasma pneumonia*, *Chlamydia pneumoniae* and *Legionella pneumophila*), *Bordetella* species causing pertussis, *Coxiella burnetii* causing Q fever, *Mycobacterium tuberculosis*, and viruses. Accordingly, culture-based methods provide limited insight into the polymicrobial community potentially present in a clinical specimen. However, culture-based methods are still preferred for the diagnosis of infectious diseases since information regarding antibiotic

susceptibility of a pathogen can directly be obtained. Hence, fast, sensitive and specific PCR-based assays are only performed for the detection of microorganisms that are very difficult to culture. Several multiplex panels are commercially available for respiratory tract infections (205-207), urogenital infections (208-211), and gastrointestinal tract Infections (212, 213). It is important to note that many multiplex PCRs are required to obtain a complete overview of a microbial community in a clinical specimen, which is practically impossible. Furthermore, PCRs can only be developed for known microorganisms.

Most recently, WGS sequencing has been introduced into a very limited number of clinical microbiological laboratories for outbreak detection of multi-drug resistant bacteria (214, 215). The fast identification of an outbreak enables a hospital or other health organisation to take preventive measures at the beginning of the outbreak to prevent the bacteria from further spreading. For example, in October 2019, the RIVM (National Institute for Public Health and the Environment) found an identical strain in several patients diagnosed with Listeria (216). A meat-slicing factory was identified as the source after comparison of the strain with strains from food and factory sampling. The concerned products were immediately recalled from the stores.

Apart from this, WGS sequencing may be useful for the identification of bacteria and to reveal the presence of antimicrobial resistance and virulence genes. The routine clinical microbiology would benefit most from this application as a case-by-case approach since there is a clear need for fast results for individual cases. Currently, WGS sequencing is most cost-effective when batches of specimens are analysed at the same time. Fast sequencing platforms that may be used in a 'per demand' may be entering the market in the near future (217). Furthermore, the correlation between genotype and phenotype remains a subject of discussion. In particular, revealing the presence or absence of antibiotic resistance genes does not always guarantee a respective phenotypic resistance or susceptibility to a specific antimicrobial drug. Another common argument for WGS sequencing never completely replacing culture-based methods is the need of an isolated pathogen for genomic input material.

16S rRNA GENE PROFILING IN THE CLINICAL MICROBIOLOGY

Compared to WGS sequencing, 16S rRNA gene profiling is not dependent on culture since it uses amplicons of total extracted DNA as input material. As a result, 16S rRNA gene profiling does not only give information regarding pathogens that are known and can be effectively cultivated but leads to a comprehensive overview of the microbial community present in a clinical specimen. This overview might be very valuable for the routine clinical microbiology for treatment decisions (218). In addition, our improved understanding of the human microbiota and its association with disease have led to the considerable need

in improved diagnostics and therapies. For adaptation of high-throughput sequencing methods in the clinical microbiology, further development of the sequencing methodology and analysis software is required (217). In particular, to shorten the turnaround time for the library preparation and the runs on the sequencing platforms, and, at the same time, further reduction of the costs. Automated pipelines for data analysis and easy-to-use software for analysis should be developed. Another key factor for adaptation of 16S rRNA gene profiling in the clinical diagnostic microbiology includes defining the clinical application for which 16S rRNA gene profiling should be considered and the evidence concerning the added value of this method. Additionally, cut-off values for interpretation of the sequencing data must be determined (218). Until now, 16S rRNA gene profiling has mainly been used as a research tool to study microbial associations with human health and disease. The limited number of studies exploring whether 16S rRNA gene profiling can be used in clinical microbiology to focus on the identification of clinically-relevant microorganisms in specimens that are normally depleted from bacteria (219, 220). These studies undervalue 16S rRNA gene profiling, since its added value for the clinical microbiology lies in the possibility to characterise complex microbial communities.

AIM AND OUTLINE OF THE THESIS

As it is outlined in this **chapter**, 16S rRNA gene profiling was of major importance for the current insights in microbial associations with human health and disease but might also be very valuable for the clinical microbiology. The added value of 16S rRNA gene profiling for the clinical microbiology remains undervalued. Accordingly, the studies described in this thesis aimed to define the clinical application for which 16S rRNA gene profiling should be considered. More specifically, the studies aimed to explore whether 16S rRNA gene profiling can be used as a direct diagnostic tool for identification of clinically relevant microorganisms or as an indirect tool for evaluation of diagnostic methods or therapies using specimens with complex microbial communities. In addition, we explored whether clinically relevant cut-off values for interpretation of the sequencing data could be defined. Furthermore, two studies were included that used 16S rRNA gene profiling as a research tool, exploring the human microbiota in health and disease.

In the first part of this thesis, we focused on using 16S rRNA gene profiling as a direct diagnostic tool. In routine clinical microbiology, standard identification of clinically-relevant microorganisms involved in lower respiratory tract infections is based on culture of bacteria from sputum followed by species identification with matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) technology and antibiotic susceptibility testing of the cultured putative causative microorganism. This approach is highly dependent on the efficient cultivation of known clinically relevant microorganisms. 16S rRNA gene profiling would result in a comprehensive overview of the microbial community present in a clinical specimen, meaning that the whole microbial community can be taken into account when making clinical decisions. However, the 16S rRNA gene lacks resolution for classification down to the species level and does not give crucial information about antibiotic susceptibility of a pathogen. Therefore, in **Chapter 2**, we questioned whether a stepwise approach using 16S rRNA gene profiling followed by species-specific qPCRs and/or culture has the potential to be a more accurate and efficient diagnostic approach than culture alone.

In the second part of this thesis, we focussed on using 16S rRNA gene profiling as an indirect tool for the clinical microbiology. First, we focussed on using 16S rRNA gene profiling as an alternative reference test for the diagnosis of bacterial vaginosis (BV). BV is characterized by a shift from *Lactobacillus* spp. dominated vaginal microbiota to a more diverse microbiota dominated by anaerobes such as *Gardnerella vaginalis*. In **Chapter 3**, we used 16S rRNA gene profiling as an alternative reference test for independent analysis of the performance of the different diagnostic methods for BV, including the current gold standard. Furthermore, BV is the main cause of base abnormal vaginal discharge, but not

the only possible cause. For the clinician, it is recommended to base diagnosis on clinical symptoms and signs, and bedside tests, supported by laboratory test findings. In **Chapter 4**, we used again 16S rRNA gene profiling as an alternative reference test to determine the best and most cost-effective algorithm based on clinical symptoms and signs, bedside and/or laboratory test findings for the diagnosis of abnormal vaginal discharge in a clinical setting. In retrospect, we determined whether implementation of the developed algorithm would have reduced the number of patients that returned to their physician with persistent symptoms due to incorrect diagnosis. In **Chapter 5**, 16S rRNA gene profiling was used to evaluate the effect of the antibiotic therapy for BV on the vaginal microbiota composition. This application was further exploited in **Chapter 6**. Here, weekly collected skin swabs of patients with mild to moderate atopic dermatitis (AD) were subjected to 16S rRNA gene profiling to analyse the inter-patient and intra-patient variability of lesional skin microbiota over time. Atopic dermatitis is associated with colonization of the skin by *Staphylococcus aureus* and a reduced microbial diversity of the skin microbiota. This analysis investigated the potential use of skin microbiota as a biomarker for clinical trials, determining the effect of an AD treatment on the skin microbiota.

In the third part of this thesis, we use 16S rRNA gene profiling as a research tool to study the link between microbiota and two different diseases: respiratory tract infections (RTIs) and male genital lichen sclerosus (MGLSc), which is chronic lichenoid inflammatory, scarring dermatosis associated with penile cancer. RTIs remain one of the leading causes of morbidity and mortality worldwide. The populations at risk are the very young (< 5 years) and the elderly (≥ 65 years). For the very young, specific upper airway microbiota profiles have been associated with increased rates of RTIs. In the elderly, the mechanisms of the heightened susceptibility to RTIs are still poorly understood. Accordingly, in **Chapter 7**, we explored whether nasal and/or oropharyngeal microbiota profiles are associated with age and RTIs in adults. In contrast to RTIs, the aetiology of MGLSc is unknown. In **Chapter 8**, we studied the microbiota of the balanopreputial sac and urine in patients with MGLSc since microbial dysbiosis may account for unresolved questions in MGLSc, about the exact nature of the relationship between urine and epithelial susceptibility, and the pathways from lichenoid inflammation to fibrosis and carcinogenesis.

Finally, the results from these studies are summarized and discussed in **Chapter 9**.

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