16S rRNA GENE PROFILING

Direct and indirect applications
for clinical microbiology

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TABLE OF CONTENTS

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**).

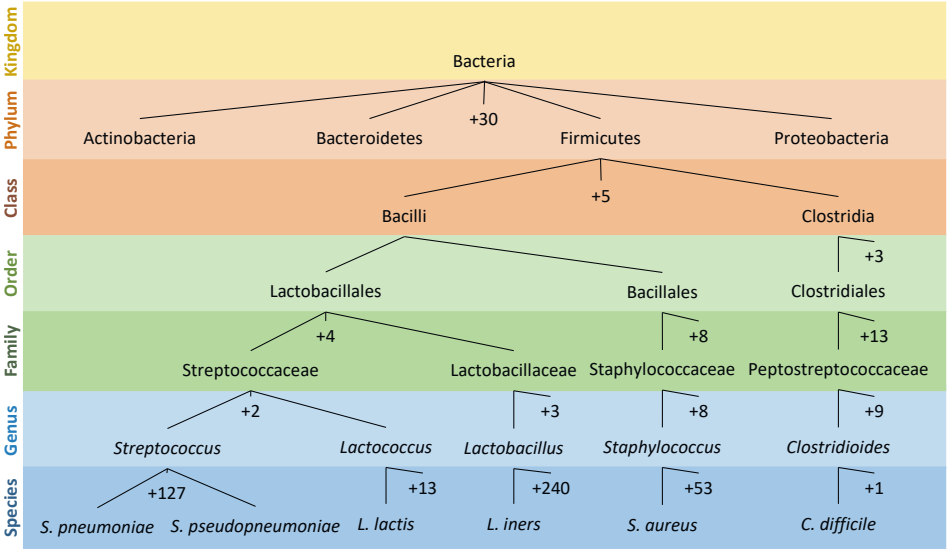

Figure 1. Bacterial taxonomy with bacterial species present in this thesis as examples. In the bacterial kingdom, bacteria are arranged in small but homogenous groups or taxa. Species is the basic taxonomic group. Groups of species are collected into genera. Groups of genera are collected into families, 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).

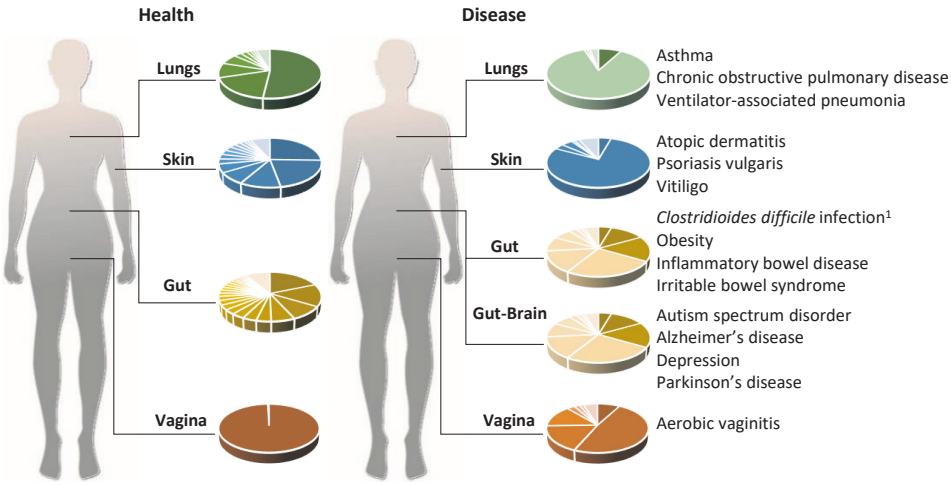

Figure 2. Examples of the Human microbiota in health and disease. Each body site, such as the gastrointestinal tract, respiratory tract, skin and urogenital system, contain a unique composition of microorganisms, called the microbiota. The microbiota composition can change in time (lose or obtain microbial 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 inflammatory 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.

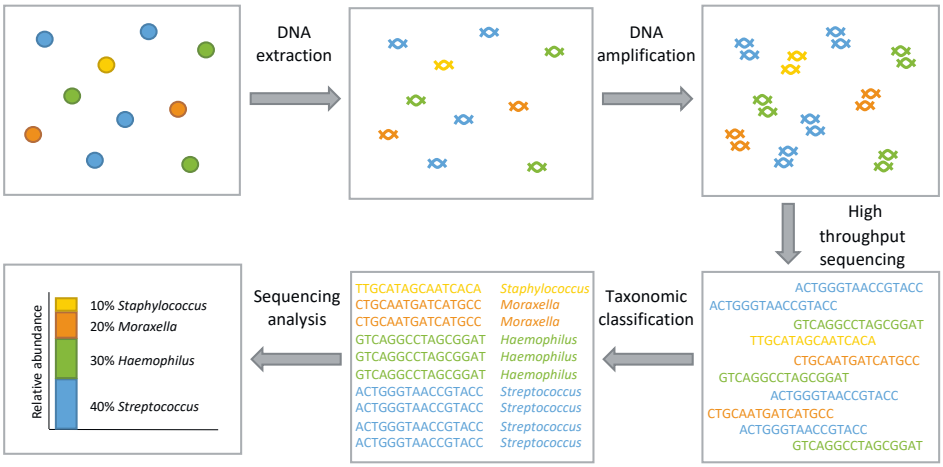

Figure 3. Flowchart of 16S rRNA gene profiling for microbiota analysis. DNA is extracted from all cells in a clinical specimen and subjected to a PCR targeting one or two regions of the 16S rRNA gene. All obtained amplicons are sequenced using a high-throughput sequencing platform. Subsequently, the 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 • Release date • Total nr of sequences • Nr of bacterial sequences	 SSU 138 Ref NR99 December 2019 510,984 431,785	 gg_13_5_99 May 2013 203,452 203,452	 Release 95 July 2019 27,212,750 22,769	 Version 16 February 2016 3,356,809 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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PART 1: DIRECT CLINICAL APPLICATION

CHAPTER 2

Evaluation of a stepwise approach using microbiota analysis, species-specific qPCRs and culture for the diagnosis of lower respiratory tract infections

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ABSTRACT

In clinical practice, the diagnosis of lower respiratory tract infections (LRTIs) is based on culture. The aim of this study was to evaluate whether a stepwise approach using microbiota analysis, species-specific quantitative real-time (q)PCRs and culture has the potential to be a more accurate and efficient diagnostic approach than culture alone. Sixty-two sputa obtained in a routine clinical setting from patients with a suspected LRTI were included. All sputa were analysed by culture, microbiota analysis based on the 16S ribosomal RNA gene and multiple species-specific qPCRs. Microbiota and culture data were compared to investigate whether cut-off values for microbiota analysis could be determined. For microbiota analysis, a relative abundance of 25% was identified as the cut-off value for the detection of both genera *Streptococcus* and *Haemophilus*. Microbiota analysis combined with species-specific qPCRs resulted in a significant increase in the number of positive sputa (73% vs 58%; $p = 0.003$) as well as in the number of identified pathogens (51 vs 37; $p = 0.049$) compared to culture. A stepwise approach using microbiota analysis, species-specific qPCRs and culture has the potential to be used in clinical settings for the diagnosis of LRTIs in the near future.

INTRODUCTION

Lower respiratory tract infections (LRTIs) are a leading cause of human morbidity and mortality worldwide (1). The standard microbiological method for identification of pathogens involved in LRTIs is culture of bacteria from sputum followed by species identification with matrix-assisted laser desorption ionization time of flight analyser (MALDI-TOF) (2) and antibiotic-susceptibility testing of the cultured putative causative microorganism. This approach is limited by poor diagnostic accuracy due to competition during selective culture and non- or poorly cultivable pathogens such as *Mycoplasma pneumonia*, *Chlamydia pneumoniae* and *Legionella pneumophila* (3). In research, accurate characterization of complex microbial communities is done by 16S ribosomal RNA (rRNA) gene sequencing (microbiota analysis). In recent years, microbial communities of different body sites have been subject of study in relation with disease. However, few studies have been performed in relation to the establishing the diagnosis of a disease.

We questioned whether a stepwise approach using microbiota analysis followed by species-specific (q)PCRs and/or culture has the potential to be a more accurate and efficient diagnostic approach than culture alone. Microbiota analysis can be used as a first screening step to divide sputa into those with and without DNA from potential pathogenic bacterial genera. Sputa without DNA from these genera can be reported as negative. The sputa with potential pathogenic genera require further analysis as a second step. The type of analysis depends on whether the potential pathogenic genus is (i) atypical (*Mycoplasma*, *Chlamydia*, *Legionella*), (ii) *Streptococcus*, *Haemophilus*, *Moraxella* or *Staphylococcus*, or (iii) others.

When an atypical genus is detected, a species-specific qPCR should be performed to discriminate between non-pathogenic species and *M. pneumoniae*, *C. pneumoniae* or *L. pneumophila*, because the 16S rRNA gene lacks sufficient resolution to allow bacterial identification lower than to the genus level (4, 5).

When the genus *Streptococcus*, *Haemophilus*, *Moraxella* or *Staphylococcus* is detected, a species-specific qPCR is required to discriminate between non-pathogenic species and *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* or *Staphylococcus aureus*. For these potential pathogenic species, quantification is required to discriminate between infection and colonization of the respiratory tract. For *S. pneumoniae*, a concentration of 1.00E+05 gene copies/mL has been described as a significant cut-off value to discriminate between infection and colonization (6). For *H. influenzae*, *M. catarrhalis* and *S. aureus*, no cut-off value has yet been determined.

When *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. aureus* or other potential pathogenic genera are detected by microbiota analysis such as Enterobacteriaceae, *Pseudomonas*, *Acinetobacter* and *Stenotrophomonas*, culture e.g. for antibiotic susceptibility testing, can be performed on indication as a third step. This step might be restricted to one selective agar plate per pathogen.

The aim of this study was to evaluate microbiota analysis combined with species-specific qPCRs for the identification of pathogens in sputum. First, data of microbiota analysis and species-specific qPCRs were compared with culture to investigate whether cut-off values for microbiota analysis as well as for the qPCRs targeting *H. influenzae*, *M. catarrhalis* or *S. aureus* could be determined.

MATERIALS AND METHODS

Study design

All sputa obtained between November 2014 and January 2015 from patients with a suspected LRTI who were hospitalized or visiting the outpatient clinic of the Haaglanden Medical Centre Bronovo hospital (The Hague), Alrijne hospital (Leiden) or Alrijne hospital (Leiderdorp, The Netherlands) were sent directly after collection to the medical microbiology laboratory for routine diagnostic analysis based on culture and MALDI-TOF. Patients were notified that remainders of their samples might be used for evaluation of diagnostic methods. If patients objected, samples were discarded. For all included sputa, microbiota analysis was performed. A qPCR assay targeting *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* or *S. aureus* was performed when the corresponding genus was identified by microbiota analysis. In case of a discrepant result between culture and microbiota analysis combined with species-specific qPCRs, microbiota analysis, species-specific qPCRs and/or whole genome sequencing was performed on the bacterial DNA harvested from the corresponding primary inoculated plates and/or subcultures.

Routine diagnostic analysis

The quality of each sputum sample was assessed by microscopic examination using Gram staining. If (polynuclear) leukocytes and alveolar cells were observed, the sputum sample was inoculated on blood, chocolate and MacConkey agar plates (Becton, Dickinson and Company, NJ, USA) and incubated for 18-24 h at 35 °C in a 5% CO_2 incubator or at 35 °C in ambient air. Subcultures were prepared for species identification by MALDI-TOF with software version 1.6.7.1000 (Bruker Corporation, Billerica, USA). Colonies morphologically suspected to be *S. pneumoniae* were tested for optochin susceptibility by Kirby-Baur disk diffusion, which was defined as an inhibition zone of ≥ 14 mm after 18 h incubation.

Pre-treatment and DNA extraction

The remaining sputum was homogenized with an equal volume of Sputasol (Oxoid Ltd, Basingstoke, UK). Bacteria of the primary inoculated agar plates as well as from the subculture plates were suspended in 2 and 1 mL cobas PCR medium (Roche Diagnostics, Meylan, France), respectively. The bacteria harvested from the culture plates were 10x

diluted in cobas PCR medium to avoid overloading of the DNA extraction system. For DNA extraction with the MagNA Pure 96 instrument, the MagNA Pure 96 DNA and Viral NA Small Volume kit (Roche Diagnostics) was used following the Viral NA Universal protocol. DNA of 200 µL sample was eluted in a final volume of 50 µL of elution buffer.

Microbiota analysis

A fragment of ~ 464bp of the V3-V4 region of the 16S rRNA gene was amplified using the primers: Bakt_341F (5'-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*CCTACGGGNGGCWGCAG-3') and Bakt_805R (5'-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GACTACHVGGGTATCTAATCC-3'). These were described by Klindworth, et al. (7) with Illumina overhang adaptor sequences added (italicized sequences). Each 50 µL PCR reaction contained 5 µL (10x) Expand High Fidelity Buffer with 15 mM $MgCl_2$ (Roche), 2.6 U Expand High Fidelity Enzyme mix (Roche), 0.2 mM of each dNTP (Roche), 0.3 µM of each primer and 10 µL of extracted DNA. The PCR was run for 2 min at 94 °C followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 1 min and a final extension step at 72 °C for 7 min. The PCR products with a visible band of ~ 531 bp on gel were subsequently purified and quantified using AMPure XP Beads (Agencourt Bioscience Corporation, Beverly, USA) and the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, UK), respectively. After library preparation using the Nextera XT kits (Illumina, San Diego, USA), sequencing was performed with the MiSeq desktop sequencer using the MiSeq Reagent Kits v2 500-cycles (Illumina). Sequencing data was processed following the QIIME pipeline. Sequences ≥ 100 bp in length with a quality score ≥ Q20 were clustered into operational taxonomic units using open reference-based approach that implements reference-based clustering following by *de novo* clustering. Clustering was conducted at a 97% similarity level using a pre-clustered version of the Augustus 2013 GreenGenes database. Unclassified reads were removed, and a sample was considered positive for a specific genus when more than 1% of the classified reads were assigned to that genus. Potential pathogenic genera included *Acinetobacter*, *Bacillus*, *Bordetella*, *Burkholderia*, *Coxiella*, *Chlamydia*, *Franciscella*, *Haemophilus*, *Legionella*, *Moraxella*, *Mycoplasma*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Staphylococcus*, *Stenotrophomonas*, *Streptococcus*, and Enterobacteriaceae (e.g. *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Serratia*, *Yersinia*). The remaining bacteria were categorised as non-pathogenic bacteria.

Quantitative real-time PCRs

qPCRs using primers and probes described previously were performed for the detection and quantification of *M. pneumoniae* (8), *C. pneumoniae* (9), *L. pneumophila* (10), *S. pneumoniae* (11), *H. influenzae* (12), *M. catarrhalis* (13) and *S. aureus* (14). Each qPCR was carried out in a total volume of 10 µL, containing 5 µL 2x SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, CA, USA) and 2 µL of extracted DNA. Amplification reactions were performed under

the following conditions: 2 min at 95 °C followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s.

The total bacterial load (16S rRNA gene) was established using a primer set (Fw 5'-GAAAGCGTGGGGAGCAAA-3', Rv1 5'-GCCGTACTCCCCAGGCGG-3' and Rv2 5'-GTCGTACT-CCCCAGGCGG-3') based on Bogaert et al. (15) and 20x EVA green (Biotium, Inc., Fremont, CA, USA). Each reaction was carried out in a total volume of 10 µL, containing 5 µL (2x) LC480 Probes Master mix (Roche Diagnostics) and 2 µL of extracted DNA. Amplification reactions were performed under the following conditions: 5 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 60 °C for 50 s and 72 °C for 1 s.

All amplification reactions were performed using a LightCycler 480 Instrument (Roche Diagnostics). For quantification, a 10-fold dilution series of a plasmid was included in each run and the second derivative analysis method was used for data analysis.

Whole genome sequencing

DNA was quantified with the Quant-iT PicoGreen dsDNA Assay Kit and a library was prepared using the Nextera XT kits. After library preparation, the PCR products were sequenced with the MiSeq desktop sequencer using the MiSeq Reagent Kits v3 600-cycles (Illumina). The bacterial species was determined by performing BLAST analysis on the with CLC bio software (QIAGEN, Aarhus, Denmark) generated consensus sequences.

Statistical analysis

McNemar and Wilcoxon signed rank tests of the statistical software package SPSS v.17.0 were used to evaluate the effect of the cut-off value on the microbiota data and to compare culture with microbiota analysis combined with species-specific qPCRs for identification of pathogens in sputum.

Data availability

The datasets generated and analysed during the current study are available in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) repository with the accession number PRJNA518755.

RESULTS

Culture

In total 62 sputa were obtained from 57 patients, of which four were admitted to the intensive care unit (ICU) (**Supplementary Table S1**). Of the 62 sputa, 26 (42%) were collected after antibiotic treatment had been started. In total 37 pathogens were identified from 36 sputa by the routine diagnostic approach based on culture (**Table 1**). No bacterial pathogens were identified in the remaining 25 sputa.

Determination of cut-off values for microbiota analysis

First, microbiota and culture data were compared to investigate whether cut-off values for microbiota analysis could be determined. In total 110 potential bacterial pathogenic genera were identified from all 62 sputa by microbiota analysis (**Table 1, Supplementary Table S2**). The most frequently identified potential pathogenic genera by microbiota analysis were *Streptococcus* and *Haemophilus* in 50/62 (81%) and in 31/62 (50%) sputa, respectively. In contrast, a *Streptococcus* sp. was identified as pathogen by culture in only 3/62 (5%) sputa and a *Haemophilus* sp. in 14/62 (23%) sputa. Comparison of the relative abundance of these genera between culture-positive and culture-negative sputa showed that all culture-positive sputa had a relative abundance > 25% for *Streptococcus* or *Haemophilus*, except for three sputa (relative abundance of 7-13% for *Haemophilus*). However, these three sputa were culture-positive for *Haemophilus parainfluenzae*, a species which role in RTIs remains unestablished. By using this cut-off value, the total number of *Streptococcus* and *Haemophilus* positive sputa reduced significantly from 50 to 25 ($p < 0.001$, McNemar test) and from 31 to 13 ($p < 0.001$, McNemar test), respectively.

Table 1. Number of bacterial pathogens identified by culture and microbiota analysis in 62 sputa

Genus	Number of identified bacterial pathogens				
	Total culture positive	Microbiota analysis without a cut-off value		Microbiota analysis with a cut-off value of 25% for the genera *Streptococcus* and *Haemophilus*	
		Total positive	Microbiota analysis negative and culture positive	Total positive	Microbiota analysis negative and culture positive
Acinetobacter	0	1	0	1	0
Enterobacteriaceae	7	8	1	8	1
Haemophilus	14	31*	1[1]	13*	41
Moraxella	4	8	0	8	0
Mycoplasma	0	2	0	2	0
Proteus	1	0	1	0	1
Pseudomonas	2	4	0	4	0
Staphylococcus	4	3	2	3	2
Stenotrophomonas	2	3	0	3	0
Streptococcus	3	50**	0	25**	0
Total	37	110	5	66	8

[1] *Haemophilus parainfluenzae* only

*/**Total positive sputa reduced significantly ($p < 0.001$, McNemar test) by using a cut-off value of 25% for microbiota analysis

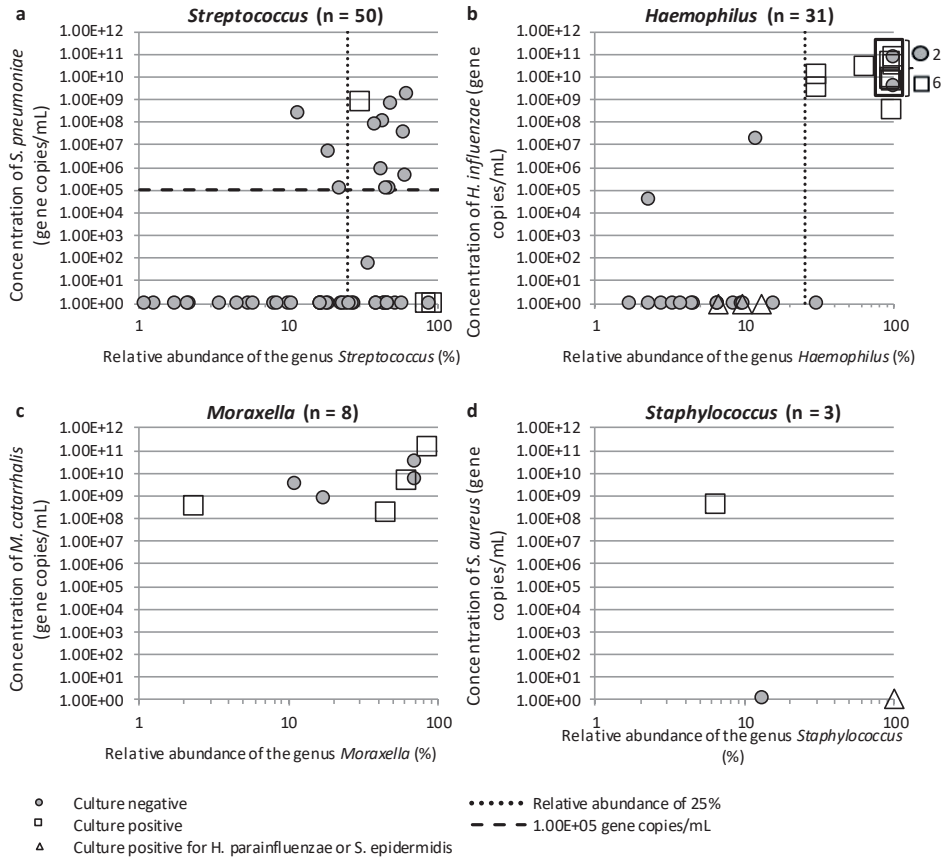

Figure 1. Culture and qPCR data of the sputa positive by microbiota analysis for the genera (**a**) *Streptococcus*, (**b**) *Haemophilus*, (**c**) *Moraxella* and/or (**d**) *Staphylococcus*. The sputa negative by qPCR are situated on the horizontal axis.

Determination of cut-off values for species-specific qPCRs

Before microbiota analysis combined with species-specific qPCRs could be evaluated for the identification of pathogens in sputum, the qPCRs targeting *H. influenzae*, *M. catarrhalis* and *S. aureus* were compared with microbiota analysis and culture to determine whether cut-off values were required to discriminate between infection and colonization. For *S. pneumoniae*, a concentration of 1.00E+05 gene copies/mL has been described as a significant cut-off value to discriminate between infection and colonization (6). Of the 25 sputa with a relative abundance ≥ 25%, 10 (40%) were positive for *S. pneumoniae* by qPCR. Nine (90%) of these 10 sputa contained a concentration of *S. pneumoniae* above this significant cut-off value of which one was also culture-positive (**Figure 1a**). Two other culture-positive sputa were negative for *S. pneumoniae* by qPCR.

For *Haemophilus*, 12/13 (92%) sputa with a relative abundance ≥ 25% were positive by qPCR (**Figure 1b**). All 12 sputa contained a relatively high concentration of *H. influenzae* ranging from 3.35E+08 to 7.45E+10 gene copies/mL, including all culture-positive sputa.

The genus *Moraxella* was identified in eight sputa by microbiota analysis and all eight sputa, including all culture-positive sputa, were positive for *M. catarrhalis* by qPCR (**Figure 1c**). All contained a relatively high concentration of *M. catarrhalis* ranging from 1.76E+08 to 1.23E+11 gene copies/mL.

The genus *Staphylococcus* was detected in three sputa by microbiota analysis and one of them was found positive for *S. aureus* by qPCR (**Figure 1d**). One of the remaining two sputa was obtained from a patient located on the ICU and the pathogen was identified by culture as *Staphylococcus epidermidis*, which is rarely pathogenic. These data indicated that species determination and quantification by qPCR for the genera *Haemophilus*, *Moraxella* and *Staphylococcus* was of no additional value in this study, which did not change by normalisation of the qPCR data using the total bacterial DNA load (**Supplementary Figure S3**).

Evaluation of microbiota analysis combined with species-specific qPCRs

For evaluation of microbiota analysis combined with species-specific qPCRs for identification of pathogens in sputum, the stepwise approach as depicted in **Figure 2** was

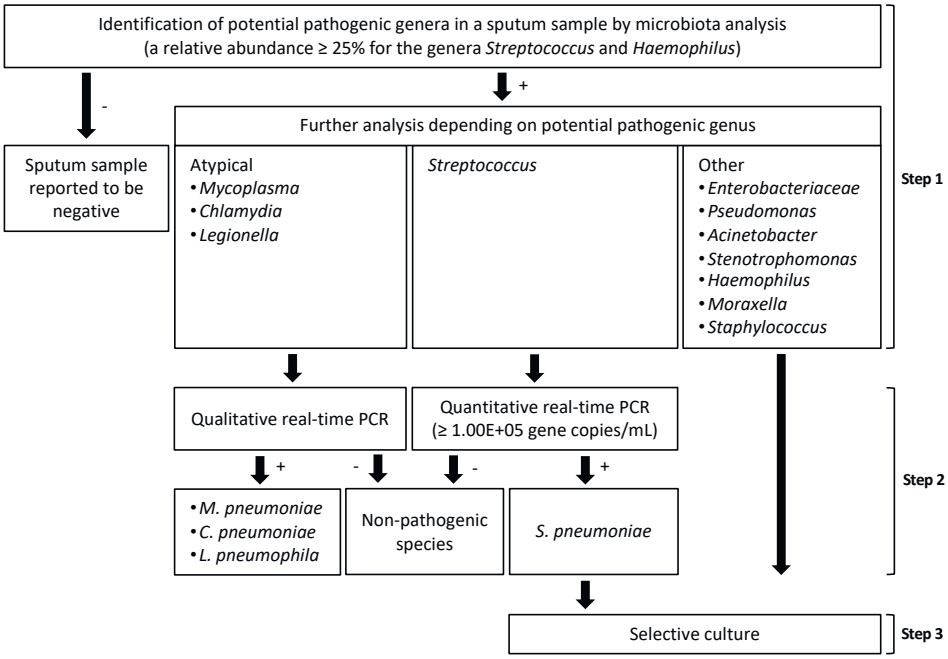

Figure 2. Final stepwise approach using microbiota analysis, species-specific qPCRs and culture for the diagnosis of lower respiratory tract infections

used. This approach included microbiota analysis with a cut-off value of 25% for the genera *Streptococcus* and *Haemophilus*, and species-specific qPCRs targeting atypical pathogens and *S. pneumoniae*. This resulted in identification of 51 pathogen in 45/62 (73%) sputa, which was a significant higher number of pathogens (p = 0.003, Wilcoxon signed rank test) and positive sputa (p = 0.049, McNemar test) compared to the 37 pathogens identified by culture in 36/62 (58%) sputa (**Figure 3**). Culture and microbiota analysis combined with species-specific qPCRs were in agreement in 36/62 (58%) sputa. In the remaining 26 sputa, 10 additional pathogens were identified by culture in 10 sputa, and 24 additional pathogens were identified by microbiota analysis combined with species-specific qPCRs in 22 sputa.

As discrepancy analysis for those bacteria, DNA harvested from the primary inoculated agar plates and subcultures were analysed by microbiota analysis, qPCR and/or whole genome sequencing. Of the 10 additional pathogens detected by culture, eight were detected by microbiota analysis (and qPCR) in the harvested bacterial DNA (**Supplementary Table S4**). These data indicated that these bacteria were present in the sputum but were detected by microbiota analysis only after selective culture had increased their relative abundance. The remaining two pathogens were misidentified in the clinical laboratory as *S. pneumoniae* instead of *S. pseudopneumoniae*.

Of the 24 additional bacteria identified as pathogen by microbiota analysis combined with qPCR, one was non-cultivable in the clinical laboratory, two were probably rarely pathogenic species and 13 were present on the agar plates but not recognized or reported as positive by the technician in the clinical laboratory (**Supplementary Table S5**). The remaining eight pathogens were only detected in the sputum sample and not in the harvested DNA by microbiota analysis and/or species-specific qPCRs.

DISCUSSION

To the best of our knowledge, this is the first study showing that a stepwise approach using microbiota analysis, species-specific qPCRs and culture has the potential to be a more accurate and efficient diagnostic approach for the diagnosis of LRTIs than culture alone. A significant higher number of pathogens and positive sputa were identified using microbiota analysis combined with species-specific qPCRs.

This study provides also a cut-off value for microbiota analysis for both the genera *Streptococcus* and *Haemophilus*. This cut-off value reduced the number of unnecessary species-specific qPCRs to be performed significantly, without missing sputa positive for *S. pneumoniae* or *H. influenzae* by culture.

Since high abundances of non-pathogenic *Streptococcus* spp. can be present in the lower respiratory tract, a next step with a species-specific qPCR was required to identify *S. pneumoniae* positive sputa (16-18). In addition, quantification of *S. pneumoniae*

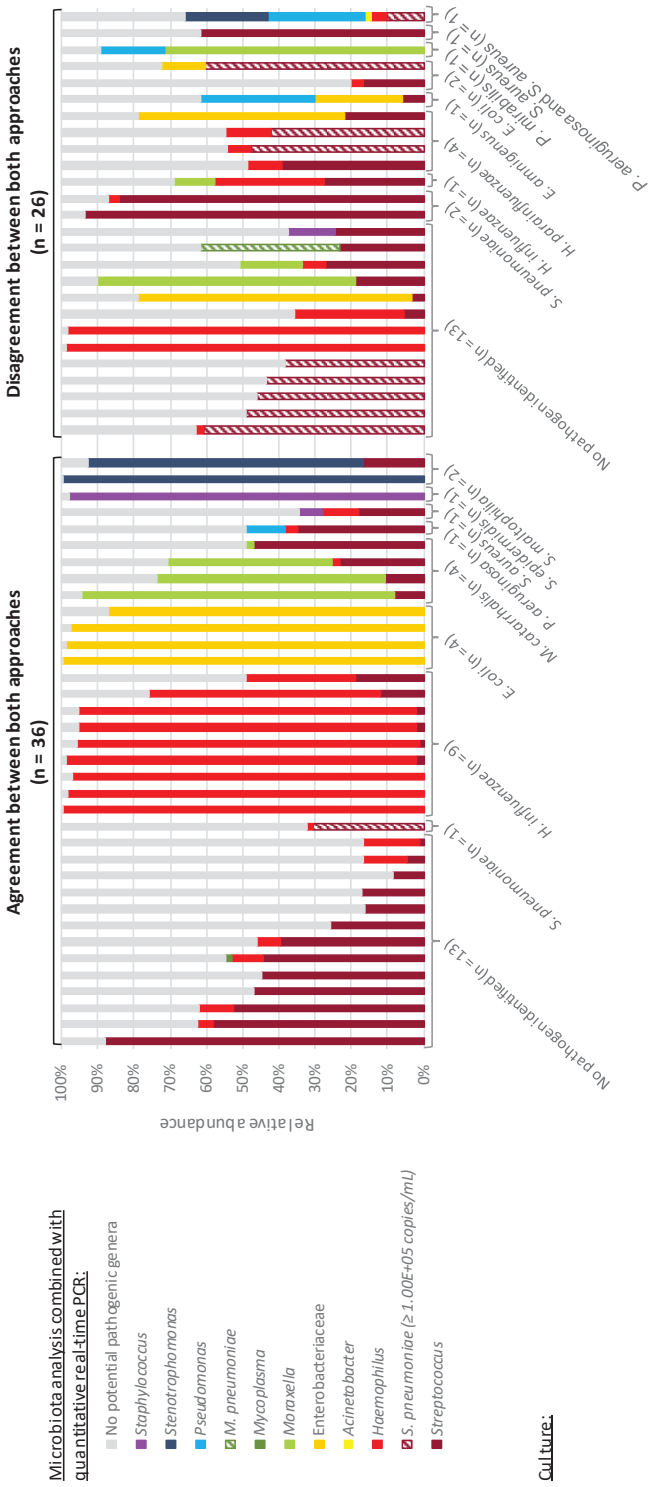

Figure 3. Comparison of culture with microbiota analysis combined with species-specific qPCR for identification of pathogens in 62 sputa. For microbiota analysis, a relative abundance of 25% for the genera *Streptococcus* and *Haemophilus* was used as cut-off value. For the *S. pneumoniae* qPCR, a concentration of 1.00E+05 gene copies/mL was used as cut-off value.

was required to discriminate between infection and colonization. Application of the widely accepted concentration of 1.00E+05 gene copies/mL as cut-off value resulted in categorization of one *S. pneumoniae* positive sputum sample as colonization (6).

For the genus *Haemophilus*, the data showed that a relative abundance of the genus *Haemophilus* above the cut-off value was only present in a sputum sample when *H. influenzae* was involved in a LRTI. Therefore, species determination and quantification by qPCR was of no additional value.

Species determination was also of no additional value for the genus *Moraxella*. All sputa positive for the genus *Moraxella* by microbiota analysis were positive and contained relatively high concentrations of *M. catarrhalis* according to qPCR. These data confirmed that potential pathogenic species colonizing the nasopharynx are only present in the lower respiratory tract when involved in a LRTI (16-18). However, this could not be confirmed for the genus *Staphylococcus* as there were only three positive sputa by microbiota analysis.

Comparison of culture and microbiota analysis combined with species-specific qPCRs for identification of pathogens revealed that clinically relevant bacteria are frequently missed by culture. This may be caused by the following: (i) the presence of uncultivable pathogens, (ii) the presence of pathogens with similar morphologic characteristics as non-pathogenic colonizers making their recognition by technicians difficult, (iii) the presence of slow-growing or fastidious pathogens overgrown by faster growing bacteria or (iv) the use of antibiotics prior to sample collection, which can have a substantial effect on the growth and subsequent identification of pathogens by culture (19-21). Only in four cases, the pathogen identified in sputum by microbiota analysis combined with species-specific qPCRs was not present on the corresponding primary inoculated agar plates and no antibiotics were used prior to sample collection. For these cases, the inhomogeneity of the sputum might explain the absence of these pathogens on the primary inoculated agar plates.

A stepwise approach using microbiota analysis, species-specific qPCRs and culture may be more efficient since less cultures are needed, because a part of the sputa can directly be reported to be negative after microbiota analysis and species-specific qPCRs have been performed. For the remaining sputa, selective culture can be prepared on indication, since the pathogen has already been identified by microbiota analysis combined with species-specific qPCRs. In this study, performing culture based on the outcome of microbiota analysis combined with species-specific qPCRs would have resulted in inoculation of 50 agar plates instead of at least 173.

The major disadvantage of this stepwise approach is that microbiota analysis is still expensive and has a turnaround time of 48 h. However, in contrast to culture, faster, better and cheaper technology for microbiota analysis can be expected in the near future. For example, the relatively new MinION sequencing platform is faster than the most widely used MiSeq sequencing platform but still has a relatively high error rate (22).

In literature, multiple molecular approaches have been described for the diagnosis of LRTIs. Approaches showing promising results were based on multiplex qPCRs (23), a DNA microarray (24) or metagenomics (25). The disadvantage of using only multiplex qPCRs or a DNA microarray is the limited number of pathogens that can be targeted at once. Rarely pathogenic bacteria (e.g. *S. epidermidis*) are not targeted by these assays and will be missed. This is not the case for metagenomics, which enables identification of all bacteria down to (sub)species level in a sputum sample. The challenge of metagenomics would be to discriminate between colonization and infection since it does not give quantitative information. Another topic of discussion is the ethical diagnosis of the human DNA sequences that will be obtained with metagenomics (26).

A limitation of the current study is that it is a heterogeneous group of patients with different LRTIs, different comorbidities, and frequently chronic lung diseases. The study was not restricted to patients with pneumonia confirmed by X-ray. Another limitation of the study is the limited number of sputa, resulting in too low numbers of some pathogens e.g. *S. aureus*, to evaluate whether a cut-off value for microbiota analysis or species-specific qPCR could be defined. In addition, a larger sample set is required to determine the clinical relevance of the defined cut-off values for microbiota analysis. The advantage of this study is that it was performed on unselected clinical samples representative for a routine clinical setting.

In conclusion, the stepwise approach using microbiota analysis, species-specific qPCRs and culture has the potential to be a more accurate and efficient diagnostic approach than culture alone for the diagnosis of LRTIs. It has the potential to be used in clinical settings for the diagnosis of LRTIs in the near future when challenges of the cost of microbiota analysis are overcome.

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SUPPLEMENTAL APPENDIX

Supplementary information accompanies this paper at
https://link.springer.com/article/10.1007%2Fs10096-019-03511-4.

PART 2: INDIRECT CLINICAL APPLICATIONS

CHAPTER 3

Comparison of Amsel criteria, Nugent score, culture and two CE-IVD marked quantitative real-time PCRs with microbiota analysis for the diagnosis of bacterial vaginosis

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ABSTRACT

Bacterial vaginosis (BV) is a common gynaecological condition. Diagnosis of BV is typically based on Amsel criteria, Nugent score and/or bacterial culture. In this study, these conventional methods and two CE-IVD marked quantitative real-time (q)PCR assays were compared with microbiota analysis for the diagnosis of BV. Eighty women were evaluated for BV during two sequential hospital visits by Amsel criteria, Nugent score, culture, the AmpliSens® Florocenosis/Bacterial vaginosis-FRT PCR kit (InterLabService, Moscow, Russia), and the BD MAX™ Vaginal Panel (BD Diagnostics, MD, USA). Microbiota analysis based on amplicon sequencing of the 16S ribosomal RNA gene was used as reference test. The microbiota profile of 36/115 (31%) included cases was associated with BV. Based on microbiota analysis, the sensitivity of detecting BV was 38.9% for culture, 61.15% for Amsel criteria, 63.9% for Nugent score and the BD MAX assay, and 80.6% for the AmpliSens assay, while the specificity of all methods was ≥ 92.4%. Microbiota profiles of the cases with discrepant results between microbiota analysis and the diagnostic methods were variable. All five diagnostic methods missed BV positive cases with a relatively high abundance of the genus *Alloscardovia*, *Bifidobacterium*, or *Dialister*, which were categorised as unspecified dysbiosis by the AmpliSens assay. Compared to Amsel criteria, Nugent score, culture, and the BD MAX assay, the AmpliSens assay was most in agreement with microbiota analysis, indicating that currently, the AmpliSens assay may be the best diagnostic method available to diagnose BV in a routine clinical setting.

INTRODUCTION

Abnormal vaginal discharge is the commonest reason why women of reproductive age consult their general practitioner for a gynaecological complaint (1). The most common cause is bacterial vaginosis (BV), which accounts for 22-50% of vaginal infectious morbidity (2). BV is a polymicrobial syndrome of unknown aetiology, characterised by a shift from *Lactobacillus* dominated vaginal microbiota to a more diverse microbiota dominated by anaerobes such as *Gardnerella vaginalis* and *Atopobium vaginae*. BV is associated with a number of adverse sequelae in obstetrics and gynaecology, including increased susceptibility to sexually transmitted infections and preterm birth (3). In 2017, the FDA recognised BV as a serious or life-threatening condition, which permitted 'Qualified Infectious Disease Products' to treat BV for 'Fast Track Designation' through the 2012 US Gain Act (4).

European guidelines recommend to base diagnosis on clinical symptoms and signs supported by additional test findings (5). Often, Amsel's clinical criteria (6), Nugent score (7), or culture-based techniques are used. According to Amsel, diagnosis of BV is based upon the presence of three out of four of the following clinical criteria: (i) vaginal pH > 4.5; (ii) homogenous white/grey adherent vaginal discharge; (iii) the presence of clue cells (vaginal epithelial cells covered in bacteria), and (iv) a positive whiff test (fishy odour after addition of potassium hydroxide). Although useful clinically as an immediate office-based test, assessment of the Amsel criteria is subjective, irreproducible, time-consuming and unpleasant to perform (8, 9). Nugent score is a Gram stain scoring system, based on the quantitative assessment of *Lactobacillus*, *Gardnerella*, and *Mobiluncus* morphotypes. It is more objective and reproducible than diagnosis based on Amsel criteria but requires a certain level of experience (9). Using culture-based techniques, BV is often diagnosed when *G. vaginalis* is isolated, but the sensitivity and specificity of this method is poor (10).

Recently, molecular-based assays became available for the diagnosis of BV, including two CE-IVD marked multiplex, quantitative (q)PCR assays (11-14). One is the AmpliSens® Florocenosis/Bacterial vaginosis-FRT PCR kit of InterLabService (henceforth referred to as AmpliSens assay), which uses the relative concentration of *Lactobacillus* spp., *G. vaginalis* clades-1 and -2, *A. vaginae* and total bacteria to diagnose BV. The other is the BD MAX™ Vaginal Panel of BD Diagnostics (henceforth referred to as BD MAX assay), which targets *Lactobacillus crispatus* and *Lactobacillus jensenii*, *G. vaginalis*, *A. vaginae*, Bacterial Vaginosis Associated Bacteria-2 (BVAB-2) and *Megasphaera*-1 for the diagnosis of BV. Both qPCR assays are fast and have a high sensitivity and specificity (15-17).

Of these additional tests, the Nugent score is considered as the gold standard for the diagnosis of BV. Another reference method is required to compare all conventional methods and qPCR assays with each other, such as 16S ribosomal RNA (rRNA) gene amplicon sequencing (microbiota analysis). This method enables accurate characterisation of complex microbial communities in terms of membership and their relative abundance to one

another. Investigation of the vaginal microbiota has shown that < 50% relative abundance of *Lactobacillus* is associated with BV (18-22). Based on statistical analysis of the vaginal microbiota data, BV has been defined as ≤ 47% relative abundance of *Lactobacillus* and increased presence of anaerobes (23). Although recommended by some, microbiota analysis is currently too laborious and expensive to be used in the routine clinical setting (24).

The aim of this study was to compare Amsel criteria, Nugent score, culture, AmpliSens, and BD MAX assay with microbiota analysis for the diagnosis of BV. First, diagnostic methods were (individually) compared with microbiota analysis using microbiota analysis as reference test. Subsequently, the vaginal microbiota profiles of the cases with discrepant results between microbiota analysis and at least one of the diagnostic methods were evaluated.

MATERIALS AND METHODS

Study design

The study was approved by the local ethics board (METC Zuidwest Holland, The Hague, The Netherlands) and written informed consent was obtained from all subjects. Sixty women complaining of abnormal vaginal discharge (increased in volume, 'thick or cheesy' in consistency, malodorous, itchy causing irritation, or a different colour from the norm of that woman), visiting the Gynaecology outpatient clinic of the Haaglanden Medical Centre (The Hague, The Netherlands) between January and July 2015 were recruited to the study. To obtain a sufficient number of BV negative swabs, 20 women visiting the outpatient clinic for either a routine cervical cytology follow-up, insertion of an intra-uterine contraceptive device or a first-trimester ultrasound in pregnant women were included. Postmenopausal women or those who had received antibiotics in the previous 3 months were excluded.

At visit 1, a standardised interview and gynaecological examination were performed. Samples were collected in the following order: (i) vaginal secretions for vaginal pH; (ii) three microscopy slides (for detection of clue cells, whiff test and Gram stain); (iii) a charcoal swab for culture, and (iv) an eSwab for the AmpliSens assay, BD MAX assay and microbiota analysis. At visit 2, approximately 4 weeks after visit 1, the gynaecological examination and sample collection were repeated.

Amsel criteria

A woman was categorised as BV positive when three out of four of the following clinical criteria were present: (i) vaginal pH > 4.5 measured using pH indicator strips with a pH range from 4.0 to 7.5 (Johnson Test Papers, Oldbury, UK); (ii) homogenous white/grey adherent vaginal discharge; (iii) the presence of clue cells detected by wet-mount microscopy, and (iv) a fishy odour after addition of 10% potassium hydroxide to a microscopic slide of vaginal secretions (6). If one of the tests could not be performed, the slide was classified as indeterminate.

Culture

Culture was performed in the routine laboratory setting. Swabs were inoculated onto chocolate agar, blood agar and blood agar with polymyxin B (BD, New Jersey, USA) and incubated at 35 °C in 5% CO_2 for 24 and 48 h. A culture was reported as BV positive if *G. vaginalis* was present as a monoculture.

Nugent score

The Gram stains were analysed in a double-blind manner by two experienced cytology technicians. For the discrepancies, consensus was achieved. The Nugent score was calculated by assessing the numbers of *Lactobacillus* morphotypes (scored as 0 to 4), *G. vaginalis* morphotypes (scored as 0 to 4), and *Mobiluncus* morphotypes (scored as 0 to 2) (7). A score of 0-3 was categorised as normal flora, 4-6 as intermediate flora, and 7-10 as BV. If the quality of the slide was poor, the slide was classified as indeterminate.

DNA extraction

DNA was extracted from a 200-µL sample and eluted in a final volume of 100 µL with the MagNA pure 96 instrument using the MagNA pure 96 DNA and Viral NA Small Volume kit and the Viral NA Plasma protocol (Roche Diagnostics, Basel, Switzerland).

CE-IVD marked assays

Both the AmpliSens and the BD MAX assay were performed according to the manufacturer's instructions. For the AmpliSens assay, a predefined algorithm of the manufacturer categorised the swabs as BV negative, BV positive, intermediate, unspecified dysbiosis or indeterminate, and for the BD MAX assay as BV negative, BV positive or indeterminate.

Microbiota analysis

Microbiota analysis was performed as described elsewhere (25). Briefly, a fragment of ~ 464 bp of the V3-V4 regions of the 16S rRNA gene was amplified. Nextera XT and MiSeq Reagent Kits v2 500-cycles (Illumina, San Diego, USA) were used for library preparation and sequencing with the MiSeq desktop sequencer (Illumina), respectively. Data was processed with the Metagenomics workflow of the MiSeq Reporter v2.3 software. A sample was considered positive for a specific genus when more than 1% of the classified reads were assigned to that genus.

Based on the microbiota profiles, samples were categorised as normal vaginal microbiota (> 47% relative abundance of *Lactobacillus*), microbiota associated with BV (≤ 47% relative abundance of *Lactobacillus* and mainly anaerobes) or microbiota associated with a different vaginal infection (≤ 47% relative abundance of *Lactobacillus* and mainly aerobes) (23). For the figures containing microbiota profiles, a limited number of genera were selected representing the microbiota composition of each sample, which included genera (i) involved

in one of the diagnostic methods if detected, (ii) associated with BV and dominating microbiota profiles or (iii) involved in aerobic vaginitis. The remaining genera formed the other genera category.

Data availability

The datasets generated and analysed during the current study are available in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) repository with the accession number PRJNA524112.

Statistical analysis

For the determination of the test characteristics, cases categorised as intermediate (Amsel criteria, AmpliSens assay), unspecified dysbiosis (AmpliSens assay), or microbiota associated with a different vaginal infection (microbiota analysis) were interpreted as BV negative. Statistical analysis was performed using the software package SPSS. To compare the sensitivity between the first and second visits, we selected at each time-point the measurements which were positive according to the reference test and performed a logistic regression, with test result as dependent and visit as independent variable. Generalised estimation equations were used to estimate the coefficients and standard errors, to account for the fact that some women provided more than one sample for the study. Test characteristics of the different diagnostic methods were compared using the McNemar Test.

RESULTS

Study population

The age of the 80 women ranged from 18 to 52 years (mean 34.1 ± 8.6 years), the majority of the women were of European origin and 25 of them were treated for BV based on clinical information at visit 1 (**Supplementary Table S1**). Of the 80 women, 14 failed to attend visit 2, and data of 31 visits were excluded because of an insufficient sample volume or indeterminate outcome by at least one of the methods, resulting in 115 complete datasets (63 from visit 1 and 52 from visit 2). Based on the microbiota profiles, 73/115 (64%) cases were categorised as normal vaginal microbiota and 36/115 (31%) as microbiota associated with BV (**Figure 1, Supplementary Table S2**). The microbiota profiles of the remaining six (5%) cases were dominated by aerobes, which is associated with a different vaginal infection, namely aerobic vaginitis (AV) (26).

Comparison of the different diagnostic methods with microbiota analysis

Amsel criteria, Nugent score, culture, AmpliSens assay and the BD MAX assay were individually compared with microbiota analysis (**Supplementary Table S3**), resulting in a

sensitivity of detecting BV of 61.1% for Amsel criteria, 63.9% for Nugent score, 38.9% for culture, 80.6% for the AmpliSens assay, and 63.9% for the BD MAX assay (**Supplementary Table S4**). The specificity of all methods was ≥ 92.4%. The sensitivity of the AmpliSens assay was significantly higher than the sensitivity of the other methods ($p \leq 0.031$; McNemar Test). There was no significant difference between test characteristics based on data of visit 1 and visit 2 for any of the methods, confirming that data of both visits could be used for calculation and comparison of the test characteristics.

Comparison of all five diagnostic methods with microbiota analysis showed that 57/73 (78%) cases with a normal vaginal microbiota profile were BV negative by all five diagnostic methods (**Figure 2a**). For the remaining 16 cases, at least two diagnostic methods were in agreement with microbiota analysis. Of the 36 cases with a microbiota profile associated with BV, seven cases (19%) were BV positive by all five diagnostic methods (**Figure 2b**). The remaining 29 cases showed variable results between the five diagnostic methods. For 24 cases, at least one diagnostic method was in agreement with microbiota analysis, whereas none of the five diagnostic methods was BV positive for the other five cases.

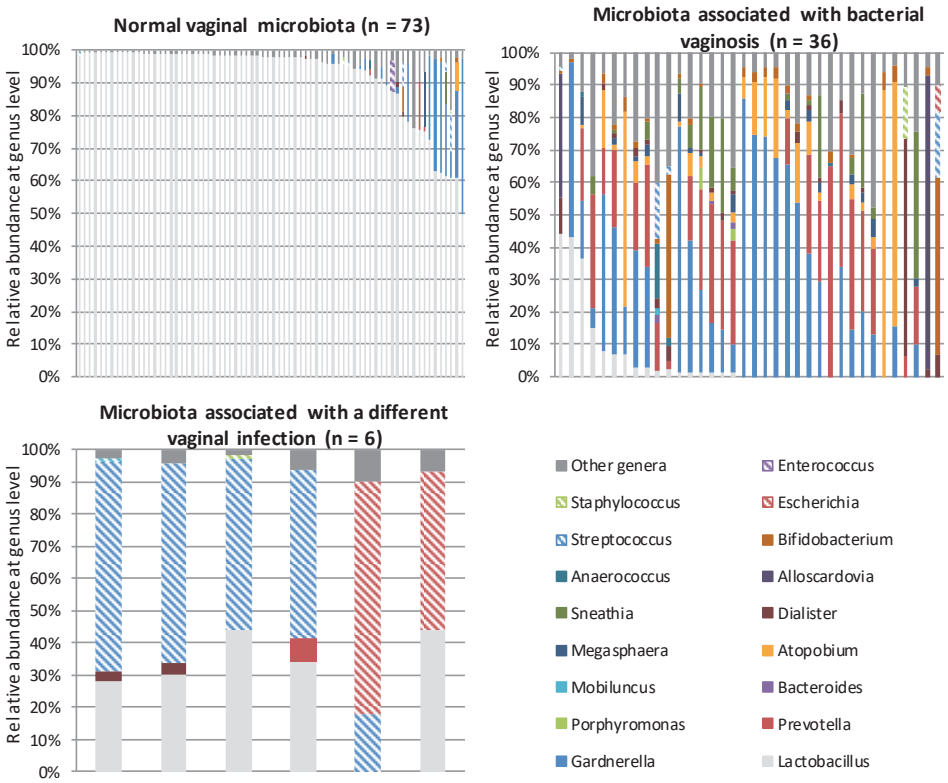


Figure 1. Microbiota profile of 115 vaginal swabs categorised as normal vaginal microbiota, microbiota associated with bacterial vaginosis or microbiota associated with a different vaginal infection

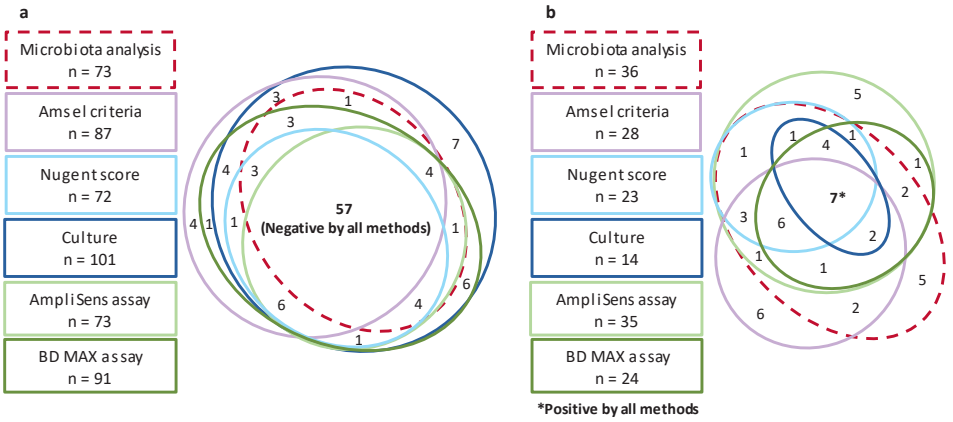


Figure 2. Venn-diagram of the number of cases categorised as (**a**) negative or (**b**) positive for bacterial vaginosis by the five different diagnostic methods and microbiota analysis

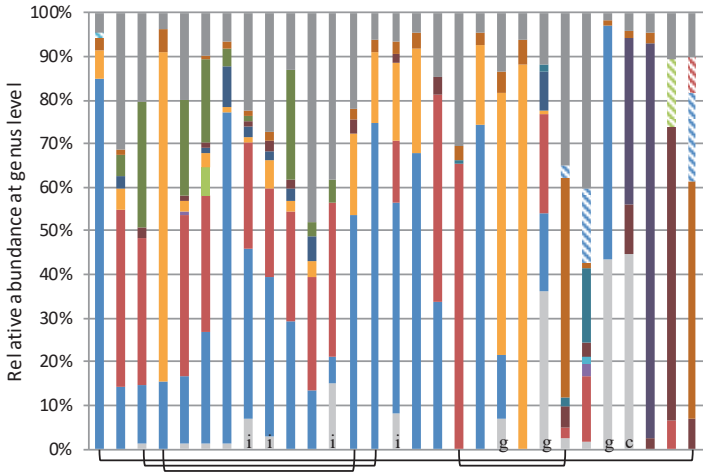
Discrepancies between microbiota analysis and the different diagnostic methods

Microbiota profiles of the swabs with discrepant results between microbiota analysis and at least one of the diagnostic methods were evaluated (**Figure 3**). Variable microbiota profiles with various dominating *Lactobacillus* spp. were observed for each diagnostic method, but all five methods missed BV positive cases that had a relatively high abundance of the genus *Alloscardovia*, *Bifidobacterium*, or *Dialister*. Three of these five cases were categorised as unspecified dysbiosis by the AmpliSens assay due to the complete depletion of *Lactobacillus* spp., and the absence of *G. vaginalis* and *A. vaginae*. The remaining two cases were categorised as BV negative due to the relatively high abundance of *Lactobacillus* spp. and/or not detecting *G. vaginalis*. Furthermore, cases categorised as intermediate by the AmpliSens assay or Nugent score had variable microbiota profiles, leaving the clinical importance of this category unknown.

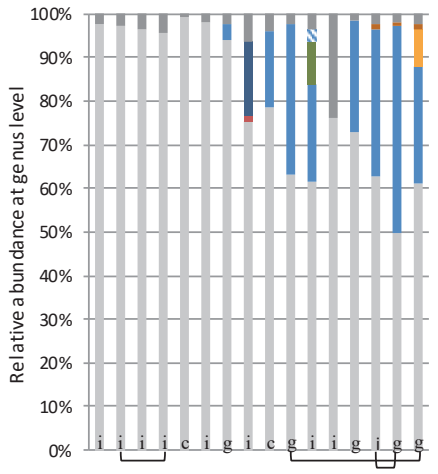
DISCUSSION

To our knowledge, this is the first study to compare Amsel criteria, Nugent score, culture, the AmpliSens and the BD MAX assay with microbiota analysis for the diagnosis of BV. Based on microbiota analysis, Amsel criteria, Nugent score, culture and the BD MAX assay each had a very low sensitivity (≤ 63.9%) compared to the AmpliSens assay (80.6%). Microbiota profiles of the cases with discrepant results between microbiota analysis and the diagnostic methods were variable, but all five diagnostic methods missed BV positive cases that had a relatively high abundance of the genus *Alloscardovia*, *Bifidobacterium* or *Dialister*.

In the present study, microbiota analysis was used as reference test because it allowed independent analysis of the performance of the different diagnostic methods, including



Microbiota analysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amsel criteria	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-
Nugent score	+	+	+	+	i	i	+	+	+	+	+	+	+	+	i	+	+	+	i	i	+	i	i	i	-	-	-	i	i
Culture	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AmpliSens assay	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	d	-	-	d	d	d	d
BD MAX assay	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Total in disagreement	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	4	4	5	5	5	5	5	5



Microbiota analysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amsel criteria	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-
Nugent score	-	-	-	-	i	i	i	i	-	-	-	i	i	i	i	i
Culture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AmpliSens assay	-	-	-	-	-	-	-	-	i	+	+	-	+	+	+	+
BD MAX assay	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Total in disagreement	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	3

Figure
- Other genera
- Enterococcus
- Staphylococcus
- Escherichia
- Streptococcus
- Bifidobacterium
- Anaerococcus
- Alloscardovia
- Sneathia
- Dialister
- Megasphaera
- Atopobium
- Mobiluncus
- Bacteroides
- Porphyromonas
- Prevotella
- Gardnerella
- Lactobacillus

c L. iners
g L. gasseri
i L. iners
⎵ Related samples

Table
+ BV positive
- BV negative
i Intermediate
d Dysbiosis unspecified

Figure 3. Microbiota profiles of the discrepancies between the five diagnostic methods and microbiota analysis

the current golden standard; Nugent score. Compared to microbiota analysis, the sensitivity of the Nugent score was low and the clinical importance of the intermediate category remains unknown. Based on these data, microbiota analysis should be considered as a serious alternative for the current golden standard to evaluate new diagnostic methods.

When all five diagnostic methods were compared to microbiota analysis, the AmpliSens assay was most in agreement with microbiota analysis. The sensitivity of 80.6%, however, remains low. One BV positive case missed by the AmpliSens assay, had a high relative abundance of *G. vaginalis*, which was probably *G. vaginalis* clades-3 or -4. Addition of these clades as targets would increase the number of BV positive samples by 3% (15). The remaining missed BV positive cases had high relative abundances of anaerobic species not targeted by the assay. Since these cases were categorised as unspecified dysbiosis, the sensitivity of the AmpliSens assay would improve if this category was interpreted as BV positive. Specificity would, however, decrease because cases with a microbiota profile dominated by aerobes are also included in this category. This is a characteristic of AV which requires different treatment than BV (27, 28). Others obtained a sensitivity of 100-96.9% for the AmpliSens assay, but a combination of Amsel criteria and Nugent score rather than microbiota analysis was used as reference test or the definition of BV was different (15, 16).

A limitation of our study is that the focus was on diagnosis of BV and therefore the diagnosis of AV was not evaluated. However, there is ongoing discussion if AV is a separate identity from BV. In this study, microbiota profiles dominated by aerobes were treated as a separate identity, which was supported by the data of the evaluated diagnostic methods.

In conclusion, compared to Amsel criteria, Nugent score, culture and the BD MAX assay, the AmpliSens assay was most in agreement with microbiota analysis. A positive or unspecified dysbiosis result is indicative of a shift in vaginal microbiota from a normal vaginal microbiota to a more diverse microbiota characterised by potentially pathogenic microorganisms. If the outcome is unspecified dysbiosis, subsequent culture should be considered to avoid missing the diagnosis of aerobic vaginitis, which requires a different treatment than BV.

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SUPPLEMENTAL APPENDIX

Supplementary information accompanies this paper at
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CHAPTER 4

Developing an algorithm for the diagnosis of abnormal vaginal discharge in a Dutch clinical setting: a pilot study

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ABSTRACT

Abnormal vaginal discharge may be caused by bacterial vaginosis, vulvovaginal candidiasis, trichomoniasis and/or aerobic vaginitis. For the development of a diagnostic algorithm, tree-based classification analysis was performed on symptoms, signs and bedside test results of 56 patients, and laboratory tests (culture, Nugent score, qPCRs) were compared. Amplicon sequencing of the 16S rRNA gene was used as reference test for bacterial vaginosis and aerobic vaginitis, culture for vulvovaginal candidiasis and qPCR for trichomoniasis. For bacterial vaginosis, the best diagnostic algorithm was to screen at the bedside with a pH and odour test and if positive, to confirm by qPCR (sensitivity 94%; specificity 97%) rather than Nugent score (sensitivity of 59%; specificity 97%; p=0.031). The analysis for the other infections was less conclusive due to the low number of patients with these infections. The developed algorithm is sensitive, specific and reduces the need for laboratory tests in 50% of the patients.

INTRODUCTION

Abnormal vaginal discharge is the most common gynaecological reason why women of reproductive age consult their general practitioner (1). Abnormal vaginal discharge may be caused by (i) bacterial vaginosis (BV; 22-50% of cases); (ii) vulvovaginal candidiasis (VVC; 17-39% of cases); (iii) trichomoniasis (4-35% of cases), (iv) aerobic vaginitis (AV; 7-12% of cases) or mixed infection (<5% of cases in the western world) (2-5). For 24-40% of the patients with abnormal vaginal discharge no cause can be found (6-8). BV and AV are both polymicrobial syndromes characterized by a shift from *Lactobacillus*-dominated vaginal microbiota to a dysbiotic microbiota dominated by anaerobes or aerobes, respectively. VVC is a fungal infection, commonly caused by *Candida albicans*, whereas trichomoniasis is a sexually transmitted infection (STI) caused by *Trichomonas vaginalis* (TV). The presence of both BV and VVC is the most common mixed infection (3). These infections are associated with a number of adverse sequelae in obstetrics and gynaecology, including increased susceptibility to sexually transmitted infections and preterm birth (9).

Misdiagnosis has been hypothesised to be the main cause for up to 40% of the patients to return to their physician with persistent symptoms after treatment (3, 5, 10, 11). Alternative reasons for therapeutic failure may be incomplete eradication of pathogens during treatment, antimicrobial or antifungal resistance, the emergence of VVC after antibiotic treatment of BV, or a STI (re)infection from an untreated or new partner (12-16).

The 2018 European International Union against Sexually Transmitted Infections (IUSTI) World Health Organisation (WHO) guideline on the management of vaginal discharge recommends diagnosing BV, VVC, TV and AV using clinical symptoms, clinical signs and bedside tests, supported by laboratory test findings (17). However, no diagnostic algorithm is proposed but instead all options are presented (**Table 1**). For BV, Gram-stained microscopy (Nugent score) (18, 19) as well as CE-IVD marked quantitative real-time PCRs (qPCRs) assays are recommended as laboratory tests (20-24).

The aim of this pilot study was to develop an algorithm to diagnose women with abnormal vaginal discharge. The first step was to determine which combination of clinical symptoms, clinical signs and bedside test results were the strongest associated with BV, VVC, TV, AV and mixed infection. The second step was to determine the best performing laboratory tests for confirmation of the diagnosis. In retrospect, it was determined whether implementation of the algorithm would have reduced the number of patients that returned to their physician with persistent symptoms due to misdiagnosis.

Table 1. Overview of clinical symptoms and signs, bedside tests results, and available laboratory tests (17)

	Bacterial vaginosis	**Vulvovaginal candidiasis**	***Trichomonas vaginalis***	**Aerobic vaginitis**
Clinical symptoms	• Malodorous discharge (fishy odour)	• Vulval itching • Vulval soreness/ irritation • Dyspareunia	• Malodorous discharge • Vulval itching • Vulval soreness/ irritation • Dysuria • Rarely lower abdominal discomfort	• Vulval soreness/ irritation • Dyspareunia
Clinical signs	• Thin white homogenous discharge coating walls of vagina and vestibule[1]	• Curdy discharge • Vulval erythema and oedema	• Yellow-green discharge • Vulval/vaginal erythema and oedema • Cervical erythema 'Strawberry cervix'	• Purulent discharge • Vaginal erythema and oedema • Vaginal ulceration
Bedside tests • Vaginal pH • Amine odour test • Wet-mount microscopy	 • >4.5[1] • Positive[1] • Clue cells[1]	 • ≤4.5 • Negative • Pseudohyphae	 • >4.5 • Positive • Flagellated protozoa	 • >4.5 • Negative • Aerobic vaginitis score[2]
Laboratory tests	• Gram-stained microscopy (Nugent score[3] or Hay Ison criteria[4]) • CE-IVD marked assays	• Culture of *Candida* spp.	• CE-IVD marked qPCR	• Culture of aerobic bacteria such as *S. agalactiae, S. aureus* and *E. coli*

[1]Amsel's clinical criteria for diagnosis of bacterial vaginosis (25).

[2]Aerobic vaginitis (AV) score combines information about *Lactobacillus* morphotypes, epithelial disruption and inflammation (26).

[3]Nugent score is based on the quantitative assessment of *Lactobacillus*, *Gardnerella* and *Mobiluncus* morphotypes (18).

[4]Hay Ison criteria is a simpler version of the Nugent score (19).

MATERIALS AND METHODS

Collection of samples and clinical data

All procedures performed were in accordance with the ethical standards of the local ethics board (METC Zuidwest Holland, The Hague, The Netherlands) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (No. 14-099; date of approval 16 January 2015). Written informed consent was obtained from all individual participants included in the study.

Sixty-four premenopausal women with complaints of abnormal vaginal discharge (increase in volume, 'thick or cheesy' in consistency, malodorous, itchy causing irritation, and/or a different colour from the norm of that woman) visiting the Gynaecology outpatient

clinic of the Haaglanden Medical Centre (The Hague, The Netherlands) between January and July 2015 were recruited for this study. At visit 1, gynaecological examination and a standardised interview with respect to clinical symptoms and signs were performed (**Table 1**). Vaginal secretions were collected for bedside and laboratory tests. Patients that did not complete the interview/gynaecological examination or had an indeterminate result for a bedside/laboratory test were excluded from the analysis. Amplicon sequencing of the 16S ribosomal RNA (rRNA) gene was used as reference test for BV/AV, culture for VVC, and qPCR for TV. Therapy was initiated according to routine hospital practice: treatment was initiated immediately if the clinical symptoms and signs were obvious, but if the clinical diagnosis was uncertain, treatment was postponed awaiting the culture results. Patients were treated according to the European guideline (27). A follow-up visit was scheduled approximately four weeks after visit 1. During this visit, clinical data and sample collection was repeated.

Bedside tests

Three bedside tests i.e., pH test, amine odour test and wet-mount microscopy, were performed by the physician. The pH test and amine odour test are part of Amsel's clinical criteria (25). pH of vaginal secretions was determined using pH indicator strips with a pH range from 4.0 to 7.5 (Johnson Test Papers, Oldbury, UK). A microscopic slide of vaginal secretions was prepared for detection of a fishy odour after addition of 10% potassium hydroxide (KOH). Another microscopic slide was prepared for detection of clue cells, pseudohyphae, and flagellated protozoa by wet-mount microscopy. The AV score was not determined (26).

Laboratory tests

Gram-stained microscopic slides were analysed to determine the Nugent score (18). Briefly, a score was generated by assessing the ratio of *Lactobacillus*, *Gardnerella vaginalis*, and *Mobiluncus* morphotypes. A score of 0-3 (normal) and 4-6 (intermediate) were interpreted as BV negative, and a score of 7-10 as BV positive. Poor quality slides were classified as indeterminate.

Culture of *G. vaginalis* for the diagnosis of BV was performed in the routine laboratory setting using vaginal secretions obtained with eSwabs as described previously (24). For the culture of yeasts, eSwabs were inoculated on Brilliance™ *Candida* Agar (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated at 35°C in ambient air. Subcultures of *Candida* spp. were prepared for species identification by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) technology (Bruker corporation, Billerica, USA). Aerobic culture for the diagnosis of AV was not performed.

For molecular methods, vaginal secretions were obtained with an eSwab. DNA isolation and microbiota analysis were performed as described previously (24). Briefly, DNA was extracted with the MagNA pure 96 (Roche Diagnostics, Basel, Switzerland). V3-V4 amplicons

of the 16S rRNA gene were sequenced with the MiSeq desktop sequencer and analysed with MiSeq Reporter software (Illumina, San Diego, USA). Based on the microbiota profiles, samples were categorised as normal vaginal microbiota (>47% relative abundance of *Lactobacillus*), microbiota associated with BV (≤47% relative abundance of *Lactobacillus* and mainly anaerobes) or as microbiota associated with AV (≤47% relative abundance of *Lactobacillus* and mainly aerobes). The extracted DNA was also used for the following CE-IVD marked qPCRs: AmpliSens® Florocenosis/Bacterial vaginosis-FRT PCR kit (henceforth referred to as BV qPCR; InterLabService, Moscow, Russia) which uses relative concentration of *Lactobacillus* spp., *Gardnerella vaginalis*, *Atopobium vaginae* and total bacteria to diagnose BV, AmpliSens® Florocenosis/*Candida*-FRT PCR kit (henceforth referred to as VVC qPCR; InterLabService) targeting *Candida albicans*, *Candida glabrata* and *Candida krusei*, *Trichomonas vaginalis* real-time PCR assay (Diagenode Diagnostics, Seraing, Belgium) and the Cobas 4800 CT/NG v2.0 test (Roche Diagnostics) and the *Mycoplasma genitalium* real-time PCR assay (Diagenode) for detection of other STIs. For diagnosis of BV, only the AmpliSens BV assay was included since we previously showed that this was the best CE-IVD marked qPCR available for the diagnosis of BV (24). All qPCRs were performed according to the manufacturer's instructions using a LightCycler 480 or Cobas 4800 Instrument (Roche Diagnostics).

Availability of data and materials

Sequencing data are available in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) repository with the accession number PRJNA524112.

Statistical analysis

For statistical analysis the software package SPSS (IBM, Chicago, IL, USA) version 25 was used. First, univariate analysis was performed to determine which symptoms, signs and bedside test results were associated with BV, VVC, TV, AV and mixed infection using the chi-squared test. Subsequently, a diagnostic algorithm to distinguish between BV, VVC, TV, AV and mixed infection was developed by building a tree-based classification model using CHAID (Chi-Squared Automatic Interaction Detection). Sensitivity and specificity of different tests were compared using the McNemar test.

RESULTS

Population characteristics

Sixty-four women complaining of abnormal vaginal discharge were recruited. The mean age of these patients was 34 years (range 18-52 years), 19 (30%) were pregnant and the majority of the patients were of European origin (**Supplementary Table S1**). Eight patients

did not complete the interview/gynaecological examination or had an indeterminate result for the pH test, leaving 56 patients for further analysis. These 56 patients were categorised as BV positive (n = 17), VVC positive (n = 7), AV positive (n = 5), mixed infection (BV and VVC; n = 3) or BV, VVC and AV negative (n = 24) using microbiota analysis as the reference test for BV and AV, and culture of *Candida* spp. for VVC. None of the patients was positive for TV according to the qPCR assay.

Determination of the best diagnostic algorithm

Step 1: screening based on clinical symptoms and signs, and bedside test results. To determine which combination of symptoms, signs and bedside test results were strongest associated with BV, VVC, AV and mixed infection, first univariate analyses were performed using microbiota analysis and yeast culture as reference test (**Supplementary Table S2**). **Table 2** summarises the data of the variables that were statistically significant associated with the different entities. A vaginal pH > 4.5 was most strongly indicative for BV, AV and mixed infection. Also, malodorous discharge, positive amine odour test, and detection of clue cells by wet-mount microscopy were significantly associated with BV. For VVC, curdy discharge and detection of pseudohyphae by wet-mount microscopy were the strongest predictors. For AV, the strongest predictors were lower abdominal discomfort and vulval/vaginal erythema and oedema. The latter was also significantly associated with mixed infection. Comparable results were obtained using the BV and VVC qPCRs as reference tests (**Supplementary Table S3**).

Subsequently, a tree-based classification analysis was performed with all variables that were significant associated with the different entities (**Supplementary Figure S4**). This multivariate analysis showed that a vaginal pH test, the amine odour test and the presence of lower abdominal discomfort was the best combination to distinguish between BV, VVC, AV and mixed infection. The presence of curdy discharge or vulval/vaginal erythema and oedema, and the detection of clue cells or pseudohyphae by wet-mount microscopy were not of added value. This screening step is the first part of the diagnostic algorithm (**Figure 1: step 1**).

Step 2: confirmation of the diagnosis using laboratory tests. According to the European guideline, the diagnosis based on the bedside tests **(Figure 1: step 1**) should be confirmed by laboratory tests (**Figure 1: step 2**). For BV, both the Nugent score and the BV qPCR are suggested as confirmation test. Bedside tests followed by qPCR as confirmation test resulted in a sensitivity of 94%, while using the Nugent score as confirmation test yielded a sensitivity of 59% (p = 0.031, McNemar test). Specificity of both was 97%. This implies that by using the Nugent score instead of the qPCR the diagnosis BV would have been missed for six patients (35%). As further note, our proposed algorithm showed significant better performance than the routine diagnostic approach of the local hospital based on clinical symptoms, signs and culture of

G. vaginalis, which is still applied in many hospitals and among general practitioners (data not shown).

For VVC, both culture and qPCR were evaluated as confirmation test. The VVC qPCR confirmed all 10 VVC culture positive samples (8 *C. albicans*, 1 *C. glabrata*, 1 *C. krusei*; mean 23.66 Ct; range 19.10-32.35 Ct), and identified three additional positive samples with a slightly higher mean Ct value of 29.55 (2 *C. albicans*, 1 *C. krusei*; 21.65-35.90 Ct). Screening followed by yeast culture or qPCR as confirmation test resulted in both cases in a sensitivity of 71% and specificity ≥ 96% (**Figure 1: step 2**).

Table 2. Clinical symptoms, clinical signs and bedside tests associated with BV, VVC, AV and mixed infection

	Microbiota analysis as reference test for BV (n = 17)		**Culture of *Candida* spp. as reference test for VVC (n = 7)**		**Microbiota analysis as reference test for AV (n = 5)**		**Mixed infection (positive for BV and VVC; n = 3)**	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Clinical symptoms								
• Malodorous discharge	**88%**	**49%**	43%	35%	60%	37%	67%	38%
• Lower abdominal discomfort	59%	44%	43%	41%	**100%**	**47%**	0%	40%
Clinical signs								
• Curdy discharge	6%	69%	**57%**	**82%**	0%	75%	33%	77%
• Vulval/vaginal erythema and oedema	12%	77%	29%	82%	**60%**	**84%**	**67%**	**83%**
Bedside test results								
• Vaginal pH > 4.5	**94%**	**62%**	29%	41%	**100%**	**49%**	100%	47%
• Positive amine odour test	**94%**	**69%**	29%	47%	20%	47%	33%	49%
• Detection of clue cells by wet-mount microscopy	**77%**	**56%**	NA	NA	NA	NA	100%	49%
• Detection of pseudohyphae by wet-mount microscopy	NA	NA	**57%**	**84%**	NA	NA	67%	81%

AV: aerobic vaginitis; BV: bacterial vaginosis; NA: not applicable; VVC: vulvovaginal candidiasis.
The bold test characteristics indicate which variables were statically significantly ($p < 0.05$) positive associated per infection using the chi-squared test.

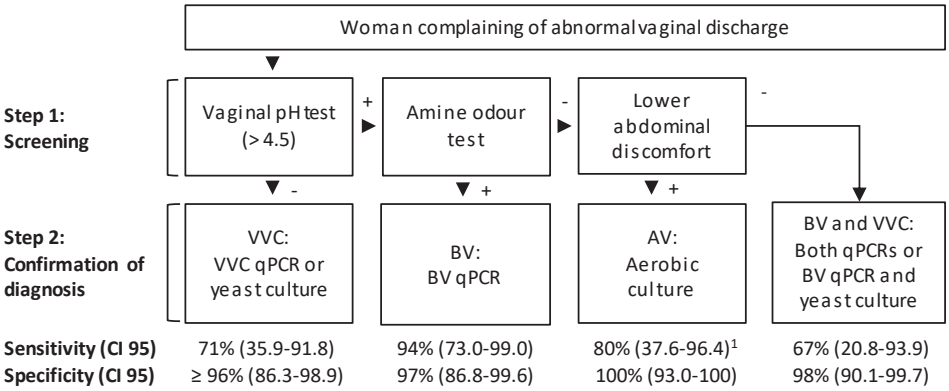

Figure 1. Best algorithm based on clinical symptoms, bedside and laboratory tests. BV: bacterial vaginosis; VVC: vulvovaginal candidiasis; AV: aerobic vaginitis; BV and VVC: mixed infection. [1]Sensitivity and specificity are calculated based on the screening results since data of aerobic culture is lacking.

For mixed infection (BV and VVC), screening followed by either both qPCRs or the combination BV qPCR and yeast culture resulted in a sensitivity of 67% and specificity of 98%. Performing standard both qPCRs or the combination BV qPCR and yeast culture would result in a sensitivity of 100% for the diagnosis of BV, VVC and mixed infection. For AV, no CE-marked qPCR is yet available leaving aerobic culture as the only confirmation test, which was not performed in this study.

Evaluation of algorithm

In retrospect, it was determined whether implementation of the algorithm as depicted in **Figure 1** would have reduced the number of patients that returned to their physician with persistent symptoms due to misdiagnosis. Eight of the 56 included patients failed to attend both visits. Of the remaining 48 patients, 27 (56%) patients returned at visit 2 with persistent symptoms. For each of these patients, microbiota (BV and AV), culture (VVC) and qPCR (VVC and STIs) data of both visits were compared (**Figure 2**). Based on this comparison, more insight in the possible cause of the persistent symptoms could be obtained. Eight (30%) of the 27 patients were misdiagnosed at the first visit (*red*). This number would have been three (11%) if our proposed algorithm was used. Treatment failure (*orange*) or the emergence of a (different) infection (*purple*) were responsible for the persistent symptoms in another eight (30%) patients. No cause was found for 10 (37%) patients (*green*) of which seven had already negative reference test results at their first visit. One pregnant patient (4%) was BV positive at her first visit but was not treated for BV as she delivered before the test results became available (*blue*). She remained BV positive after giving birth. None of the patients were positive for a STI at both visits.

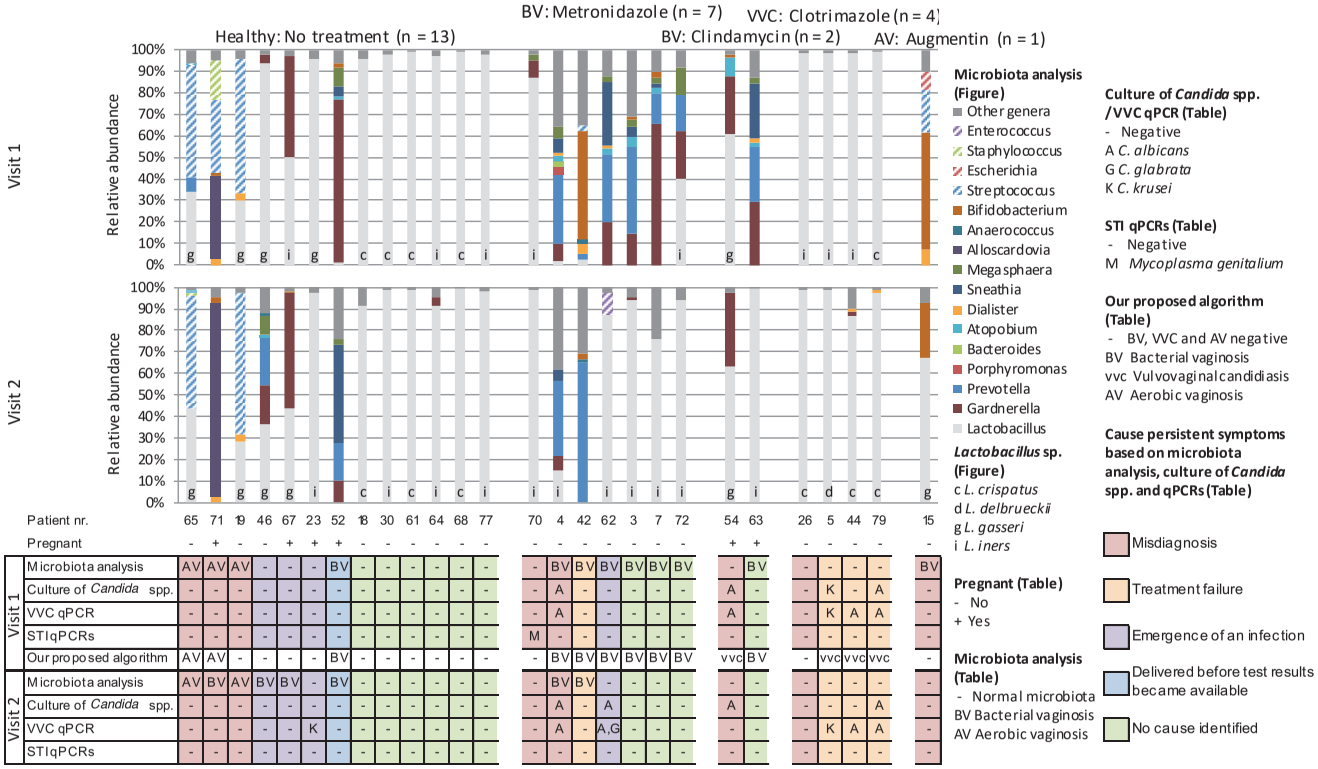

Figure 2. Retrospective evaluation of the proposed algorithm using data from 27 patients with persistent symptoms. Current treatment was initiated based on the routine diagnostic approach of the hospital using clinical symptoms, signs and culture results. Patients diagnosed with BV were treated with metronidazole or clindamycin in case of pregnancy or lactation. Patients diagnosed with VVC were treated with clotrimazole. One patient was treated with amoxicillin-clavulanic acid for a vaginal *Escherichia* coli infection. For each patient, cause of persistent symptoms was determined by comparison of data obtained during both visits. Misdiagnosis was defined as a disagreement between the test outcomes at visit 1 and the current treatment decision (*red*), treatment failure as an agreement between the positive test outcomes of both visits and the current treatment decision (*orange*), and emergence of an infection as a disagreement between the positive test outcomes of both visits (*purple*). No cause was identified when the patient was not or correctly treated and all test results of visit 2 were negative (*green*). One pregnant patient was BV positive at her first visit but was not treated for BV as she delivered before the test results became available (*blue*).

DISCUSSION

To the best of our knowledge, this is the first study developing an algorithm to diagnose women with BV, VVC, AV or mixed infection based on the clinical symptoms, clinical signs, bedside and laboratory tests as described in the European guideline. Microbiota analysis was used as reference test for BV and AV, and culture for VVC. The results of this study suggest that with a simple algorithm BV can be identified with a high degree of certainty, and the need of laboratory tests to be performed and the number of patients returning to the physician with persistent symptoms can be reduced significantly.

This study showed that none of the clinical symptoms or signs can differentiate between BV, VVC, AV and mixed infection, whereas the combination of two bedside tests (pH and amine odour test) turned out to be of diagnostic value to differentiate between BV or AV and other entities (step 1). In line with previous reports, an elevated vaginal pH was indicative for patients with a dysbiotic vaginal microbiota (26, 28-30). The amine odour test was required to differentiate between BV (BV is more likely when test positive) and AV (AV is less likely when test positive). Patients with a mixed infection of BV and VVC had an elevated pH and a negative amine odour test. The presence or absence of lower abdominal discomfort was found to differentiate between AV and mixed infection (BV and VVC positive). Patients with a normal vaginal pH had most likely VVC. However, both observations of mixed infection associated with negative amine odour test and lower abdominal discomfort differentiating AV from mixed infection were based on a low number of positive samples and should be confirmed in a larger population.

The clinical tests should be followed by a confirmation test (step 2). Patients with an elevated pH and positive amine odour test should be tested for BV, normal pH for VVC, elevated pH, negative amine odour test and presence of lower abdominal discomfort for AV and elevated pH, negative amine odour test and absence of lower abdominal discomfort for BV and VVC. The BV qPCR performed significant better as confirmation tests for BV than the Nugent score. For the detection of *Candida* spp., the test characteristics of culture and the VVC qPCR were comparable. The advantage of performing the VVC qPCR, next to the BV qPCR, is the short turnaround time and the necessity of submitting one sample only. The reasons to perform the culture rather than the VVC qPCR are the probably lower costs, detection of all yeasts and the possibility to perform susceptibility testing. For AV, only aerobic culture is available as confirmation test.

Instead of the abovementioned algorithm one could choose to routinely perform both BV and VVC qPCRs and aerobic culture for detection of AV. To reduce the number of aerobic cultures, an alternative route would be to perform aerobic culture based on the outcome 'unspecified dysbiosis' by the BV qPCR since this result is indicative for the diagnosis of AV (24). Routinely performing BV and VVC qPCR (and AV culture) make bedside tests, which are time consuming and unpleasant to perform (31, 32), redundant and provide a better

diagnostic outcome but likely increase the laboratory costs. In our study population, the result of the bedside tests indicated no BV in approximately 50% of the patients, reducing the number of diagnostic assays to be performed and associated costs by the same percentage.

The best diagnostic approach to detect TV could not be determined. Our study population lacked TV positive patients and was at low risk for STIs. We suggest testing patients who are at risk for TV and other STIs with qPCR. However, the developed diagnostic algorithm may not extrapolate to regional or racial groups with high risk for TV and other STIs. Furthermore, performing a qPCR is only feasible in resource-rich settings.

This study also provides more insight in the cause of persistent symptoms. Misdiagnosis, treatment failure and emergence of a different infection after treatment were important causes of persistent symptoms. However, for approximately 40% of the patients experiencing (persistent) abnormal vaginal discharge no cause could be found. These patients probably have physiological discharge or may suffer from other conditions, such as cervicitis, mucoid ectopy, vulval dermatoses or allergic reactions. In agreement with our findings, others reported that for 24-40% of the patients with abnormal vaginal discharge no cause could be found (6-8). Implementation of the proposed algorithm might have reduced the number of patients that returned to their physician with persistent symptoms by approximately 20%.

A limitation of this study is the small study population. The analysis of the clinical symptoms, clinical signs and bedside test results for the diagnosis of VVC, TV, AV and mixed infection were less conclusive, since each group contained less than seven positive patients. A larger study population is required to validate the proposed algorithm. Another limitation is the lack of aerobic culture and AV score data. AV is a relatively newly recognised cause of vaginal discharge, which is the reason why Aerobic culture and AV score were not included in the study design (26). In a follow-up study, these methods should be included to confirm the redundancy of the AV score in the test algorithm and the utility of the aerobic culture as confirmation test. The advantage of this study is the development of an algorithm for BV, VVC, AV and mixed infection instead of a separate algorithm for each entity using microbiota analysis as reference test for BV and AV.

CONCLUSIONS

The best algorithm to diagnose BV is to screen at the bedside with a pH test and amine odour test, and if positive, to confirm by qPCR. This is a sensitive and specific approach, and in line with the 2018 European (IUSTI/WHO) guideline. Furthermore, application of this algorithm reduces the need for laboratory tests significantly and reduces the number of patients with misdiagnosis, leading to less patients returning to the physician after treatment.

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SUPPLEMENTAL APPENDIX

Supplementary Table S1. Population characteristics

Characteristics	Women with complaints of abnormal vaginal discharge (n = 64)
Age, mean (range)	34 (18-52)
Ethnicity, n (%)	
European	51 (80)
Latin-American	3 (5)
African	2 (3)
East Asian	2 (3)
South Asian	2 (3)
Middle Eastern	2 (3)
Mixed origin	2 (3)
Use of vaginal shower gel, n (%)	5 (8)
Sexually active, n (%)	55 (86)
Number of sexual partners in the past three months, mean (range)	1 (0-1)
Anticonception, n (%)	
No anticonception	38 (59)
Anticonception pill	12 (19)
Levonorgestrel intrauterine devices	9 (14)
Condom	4 (6)
Copper intrauterine devices	1 (2)
Pregnant, n (%)	19 (30)
Breast feeding, n (%)	3 (5)
First day of last menstrual period	At least 4 days ago[1]

[1]Menstrual bleeding results in an indeterminate vaginal pH test, causing exclusion of the patient from the study

Supplementary Table S2. Evaluation of clinical symptoms, clinical signs, and bedside test results for the diagnosis of bacterial vaginosis (n = 17), vulvovaginal candidiasis (n = 7), aerobic vaginitis (n = 5) and mixed infection (n = 3) using microbiota analysis and culture of *Candida* spp. as the reference tests

	Total number of positive women (n = 56)	Microbiota analysis as the reference test for BV			Culture of *Candida* spp. as the reference test for VVC			Microbiota analysis as the reference test for AV			Mixed infection (positive for BV and VVC)		
		Sensitivity (CI 95)	Specificity (CI 95)	p[1]	Sensitivity (CI 95)	Specificity (CI 95)	p[1]	Sensitivity (CI 95)	Specificity (CI 95)	p[1]	Sensitivity (CI 95)	Specificity (CI 95)	p[1]
Clinical symptoms													
Dyspareunia[2]	unknown	Could not be determined			Could not be determined			Could not be determined			Could not be determined		
Dysuria	0 (0%)	Not applicable			Not applicable			Not applicable			Not applicable		
Malodorous discharge	35 (63%)	88% (65.7-96.7)	49% (33.9-63.8)	0.009	43% (15.8-75.0)	35% (22.9-48.7)	0.251	60% (23.1-88.2)	37% (25.3-51.0)	0.904	67% (20.8-93.9)	38% (25.9-51.2)	0.878
Low abdominal discomfort	32 (57%)	59% (36.0-78.4)	44% (29.3-59.0)	0.867	43% (15.8-75.0)	41% (28.2-54.8)	0.414	100% (56.6-100)	47% (34.1-60.5)	0.042	0% (0.0-56.0)	40% (27.6-53.1)	0.040
Vulval itching	25 (45%)	35% (17.3-58.7)	51% (36.2-66.1)	0.353	71% (35.9-91.8)	59% (45.3-71.8)	0.128	40% (11.8-77.0)	55% (41.4-67.8)	0.827	67% (20.8-93.9)	57% (43.3-69.1)	0.430
Vulval soreness/irritation	19 (34%)	29% (13.3-53.1)	64% (48.4-77.3)	0.637	43% (15.8-75.0)	67% (53.4-78.8)	0.594	60% (23.1-88.2)	69% (55.0-79.7)	0.197	33% (6.2-879.2)	66% (52.6-77.3)	0.982
Clinical signs													
Curdy discharge	13 (23%)	6% (1.1-27.0)	69% (53.6-81.4)	0.043	57% (25.1-84.2)	82% (68.6-90.0)	0.023	0% (0.0-43.5)	75% (61.1-84.5)	0.198	33% (6.2-79.2)	77% (64.5-86.6)	0.670
Purulent discharge	0 (0%)	Not applicable			Not applicable			Not applicable			Not applicable		
Thin white homogenous discharge coating walls of vagina and vestibule[3]	18 (32%)	47% (26.2-69.0)	74% (58.9-85.4)	0.115	0% (0.0-35.4)	63% (49.3-75.3)	0.052	20% (3.6-62.5)	67% (53.0-78.0)	0.542	67% (20.8-93.9)	70% (56.5-80.5)	0.188
Vulval/vaginal erythema and oedema	11 (20%)	12% (3.3-34.3)	77% (61.7-87.4)	0.327	29% (8.2-64.1)	82% (68.6-90.0)	0.525	60% (23.1-88.2)	84% (72.0-91.8)	0.017	67% (20.8-93.9)	83% (70.8-90.8)	0.035
Yellow-green discharge	20 (36%)	41% (21.6-64.0)	67% (51.0-79.4)	0.573	43% (15.8-75.0)	65% (51.3-77.1)	0.673	40% (11.8-77.0)	65% (51.0-76.4)	0.834	0% (0.0-56.2)	62% (48.8-74.1)	0.184
Bedside test results													
Vaginal pH > 4.5[3]	31 (55%)	94% (73.0-99.0)	62% (45.9-75.1)	< 0.001	29% (8.2-64.1)	41% (28.2-54.8)	0.128	100% (56.6-100)	49% (35.9-62.3)	0.035	100% (43.9-100)	47% (34.4-60.3)	0.110
Positive amine odour test[3]	28 (50%)	94% (73.0-99.0)	69% (53.6-81.4)	< 0.001	29% (8.2-64.1)	47% (33.7-60.6)	0.225	20% (3.6-62.5)	47% (34.1-60.5)	0.160	33% (6.2-79.2)	49% (36.1-62.1)	0.553
Detection of clue cells by wet-mount microscopy[3]	30 (54%)	77% (52.7-90.4)	56% (41.0-70.7)	0.023	Not applicable			Not applicable			100% (43.9-100)	49% (36.1-62.1)	0.097
Detection of pseudohyphae by wet-mount microscopy	12 (21%)	Not applicable			57% (25.0-84.2)	84% (71.0-91.5)	0.014	Not applicable			67% (20.8-93.9)	81% (68.6-89.4)	0.050

AV: aerobic vaginitis; BV: bacterial vaginosis; CI: confidence interval; VVC: vulvaginal candidiasis.

[1]Chi-squared test was used for statistical comparison

[2]Not all women could confirm the presence or absence

[3]Amsel criteria for diagnosis of BV

Supplementary Table S3. Evaluation of clinical symptoms, clinical signs, and bedside test results for the diagnosis of bacterial vaginosis (n = 14), vulvovaginal candidiasis (n = 7) and mixed infection (n = 6) using the qPCRs as the reference tests

	Total number of positive women (n = 56)	BV qPCR as the reference test for BV[1]			VVC qPCR as the reference test for VVC			Mixed infection (positive BV and VVC qPCR)		
		Sensitivity (CI 95)	Specificity (CI 95)	p[2]	Sensitivity (CI 95)	Specificity (CI 95)	p[2]	Sensitivity (CI 95)	Specificity (CI 95)	p[2]
Clinical symptoms										
Dyspareunia[3]	unknown	Could not be determined			Could not be determined			Could not be determined		
Dysuria	0 (0%)	Not applicable			Not applicable			Not applicable		
Malodorous discharge	35 (63%)	86% (60.1-96.0)	45% (31.2-60.1)	0.038	43% (15.8-75.0)	35% (22.9-48.7)	0.251	83% (43.7-97.0)	40% (27.6-53.8)	0.265
Low abdominal discomfort	32 (57%)	71% (45.4-88.3)	48% (33.4-62.3)	0.212	71% (35.9-91.8)	45% (31.9-58.7)	0.414	17% (3.0-56.4)	38% (25.9-51.9)	0.034
Vulval itching	25 (45%)	43% (21.4-67.4)	55% (40.0-68.8)	0.877	57% (25.1-84.2)	57% (43.3-70.0)	0.477	50% (18.8-81.2)	56% (42.3-68.8)	0.780
Vulval soreness/irritation	19 (34%)	36% (16.3-61.2)	67% (51.6-79.0)	0.871	43% (15.8-75.0)	67% (53.4-78.8)	0.594	33% (9.7-70.0)	66% (52.2-77.6)	0.974
Clinical signs										
Curdy discharge	13 (23%)	7% (1.3-31.5)	71% (56.4-82.8)	0.100	57% (25.1-84.2)	82% (68.6-90.0)	0.023	33% (9.7-70.0)	78% (64.8-87.3)	0.534
Purulent discharge	0 (0%)	Not applicable			Not applicable			Not applicable		
Thin white homogenous discharge coating walls of vagina and vestibule[4]	18 (32%)	50% (26.8-73.2)	74% (58.9-84.7)	0.099	0% (0.0-35.4)	63% (49.3-75.3)	0.052	33% (9.7-70.0)	68% (54.2-79.2)	0.947
Vulval/vaginal erythema and oedema	11 (20%)	14% (4.0-39.9)	79% (64.1-88.3)	0.560	14% (2.6-51.3)	80% (66.4-88.5)	0.703	50% (18.8-81.2)	84% (71.5-91.7)	0.048
Yellow-green discharge	20 (36%)	29% (11.7-54.7)	62% (46.8-75.0)	0.520	43% (15.8-75.0)	65% (51.3-77.1)	0.673	17% (3.0-56.4)	62% (48.2-74.1)	0.303
Bedside tests										
Vaginal pH > 4.5[4]	31 (55%)	100% (78.5-100)	60% (44.5-73.0)	< 0.001	29% (8.2-64.1)	41% (28.2-54.8)	0.128	67% (30.0-90.3)	46% (33.0-59.6)	0.555
Positive amine odour test[4]	28 (50%)	100% (78.5-100)	67% (51.6-79.0)	< 0.001	43% (15.8-75.0)	49% (35.6-62.5)	0.686	17% (3.0-56.4)	46% (33.0-59.6)	0.084
Detection of clue cells by wet-mount microscopy[4]	30 (54%)	79% (52.4-92.4)	55% (40.0-68.8)	0.030	Not applicable			50% (18.8-81.2)	46% (33.0-59.6)	0.853
Detection of pseudohyphae by wet-mount microscopy	12 (21%)	Not applicable			43% (15.8-75.0)	82% (68.6-90.0)	0.140	67% (30.0-90.3)	84% (71.5-91.7)	0.004

AV: aerobic vaginitis; BV: bacterial vaginosis; CI: confidence interval; VVC: vulvaginal candidiasis.

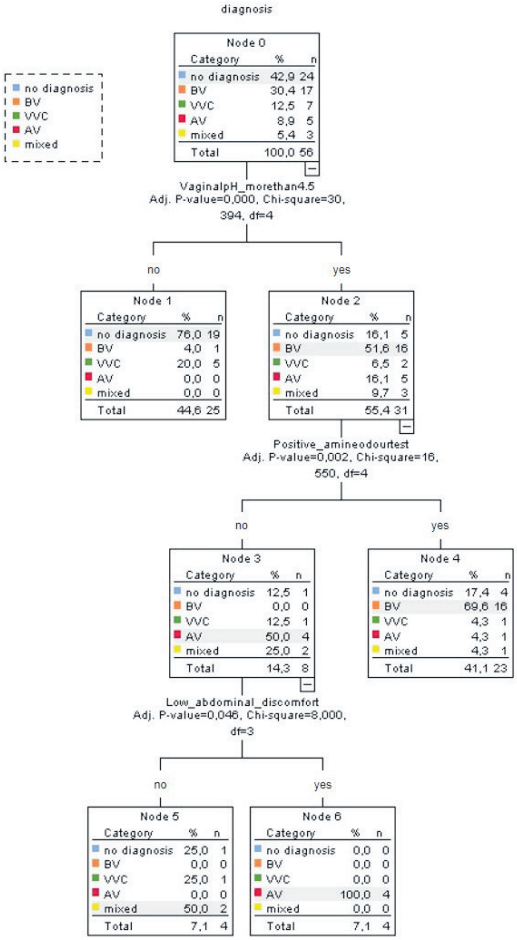
[1]Categrory BV positive

[2]Chi-squared test was used for statistical comparison

[3]Not all women could confirm the presence or absence

[4]Amsel criteria for diagnosis of BV

Supplementary Table S4. Development of diagnostic algorithm to distinguish between bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), aerobic vaginitis (AV) and mixed infection by building a tree-based classification model using Chi-Squared Automatic Interaction Detection. Microbiota analysis and culture of *Candida* spp. were used as reference tests.



CHAPTER 5

The vaginal microbiota in the course of bacterial vaginosis treatment

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ABSTRACT

Bacterial vaginosis (BV) is perceived as a condition of disrupted vaginal microbiota, but remains of unknown aetiology. In this study, vaginal microbiota composition was determined in twenty-one women with BV, before and after treatment with metronidazole or clindamycin. Microbiota composition varied greatly between women and defining a (un) healthy vaginal microbiota state remains elusive, challenging BV diagnosis and treatment. While relative abundance of *Lactobacillus* increased after antibiotic treatment in two-third of women, its abundance was not associated with treatment outcome. Instead, remaining complaints of abnormal vaginal discharge were more common after metronidazole treatment and associated with increased relative abundance of *Ureaplasma*.

INTRODUCTION

The vaginal microbiota plays a crucial role in maintaining a healthy vaginal environment and perturbation of this system has been implicated in disturbed vaginal health and other negative outcomes (1, 2). The vaginal microbiota is dynamic and influenced by hormonal changes, sexual activity and hygiene (3). Various vaginal bacterial communities exist in healthy women, mostly dominated by *Lactobacillus* species, while some are being composed of anaerobes like *Atopobium* and *Prevotella* species (4). Nevertheless, the common perception of a healthy vaginal microbiota is one dominated by one or more *Lactobacillus* species. As such, the switch from a *Lactobacillus*-dominated microbiota to a more diverse microbiota, in combination with clinical symptoms, is considered as bacterial vaginosis or aerobic vaginitis, depending on colonisation by anaerobic or aerobic bacteria, respectively. Bacterial genera that are specifically associated with BV are, amongst others, *Gardnerella*, *Atopobium*, *Prevotella*, *Fusobacterium* and *Dialister* species (5). Despite these associations, the aetiology of BV is unknown, and diagnosis and treatment remain elusive. While a Gram-stain evaluation according to the Nugent criteria is considered the golden standard for BV diagnosis, it is not routinely applied in a clinical setting (6). Instead, BV diagnosis is commonly based on clinical signs and symptoms or Amsel criteria (7). Symptoms of BV can resolve without intervention, but metronidazole or clindamycin can be prescribed in case of persistence, even though recurrence is common (8, 9). In our study, vaginal microbiota composition of women with clinically diagnosed BV was determined before and after antibiotic treatment and related to clinical characteristics.

MATERIALS AND METHODS

Prospectively, vaginal secretions and clinical data were collected from 60 premenopausal women visiting the Gynaecology outpatient clinic of the Haaglanden Medical Centre (The Hague, The Netherlands) with complaints of abnormal vaginal discharge. Vaginal secretion was collected using the ESwab (Copan Diagnostics Inc, USA). BV diagnosis was based on clinical symptoms and signs, with malodorous discharge as major criterium for diagnosis of bacterial vaginosis, followed by culturing when clinical diagnosis based on symptoms alone was uncertain. Therapy was initiated according to routine hospital practice following the European guideline and consisted of 500 mg metronidazole taken orally twice a day for seven days, or, in case of pregnancy or lactating, 300 mg clindamycin taken orally twice a day for seven days (13). A follow-up visit was scheduled approximately four weeks after inclusion, during which vaginal swab and clinical data collection were repeated. Women who were clinically diagnosed with BV and attended the follow-up visit were selected for microbiota profiling (n = 21). Clinical data collection, Amsel criteria (vaginal pH, amine odour,

wet-mount microscopy), Nugent score, and *Gardnerella vaginalis* culturing, were performed for research purposes as previously described (14). Detailed subject characteristics are outlined in **Table 1**. The Declaration of Helsinki was the guiding principle for trial execution, and the study was approved by the local ethics board (METC Zuidwest Holland, The Hague, The Netherlands). All patients provided written informed consent before participation.

Table 1. Patient characteristics

		Before treatment	After treatment
Demographics	N	21	21
	Age (mean ± SD years)	32.5 ± 7.6	32.5 ± 7.6
	European	15	15
Antimicrobials	Clindamycin	-	11
	Metronidazole	-	10
	Clotrimazole	-	4
	Azithromycin	-	1
Symptomology	Abnormal discharge	21	9
	Malodorous discharge	20	4
	Increased discharge	13	5
	Yellow/green discharge	7	2
	Curdy discharge	2	2
	Thin white discharge	8	5
	Purulent discharge	1	0
	Vulvar erythema oedema	4	2
	Vulvar itching	9	3
	Vulvar irritation	6	3
	Cervical erythema	3	2
	Cervical bleeding	1	0
	Low abdominal pain	10	3
Diagnosis	Bacterial vaginosis	21	2
	Nugent score positive	12	5
	Amsel criteria positive	13	4
	Vaginal pH > 4.5	18	12
	Amine odour	16	8
	Clue cells	14	4
Other	Anticonception	6	6
	Vaginal shower gel	0	1
	Sexually active	20	20
	Pregnant	8	8
	Lactating	3	3

SD: standard deviation.

Vaginal bacterial microbiota was determined by 16S rRNA gene amplicon sequencing of the V3-V4 region using the Nextera XT, MiSeq Reagent Kits v2 500 cycles and a MiSeq desktop sequencer (Illumina, USA). Raw sequencing data are available in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under study accession PRJNA524112. Read filtering, operational taxonomic unit (OTU)-picking and taxonomic assignment were performed using the NG-Tax 0.4 pipeline and the Silva_132_SSU Ref database (10). Statistical analysis and data visualisation were performed in R (v3.5.1) using the packages phyloseq (v1.26.1), vegan (v2.5-4), ggplot2 (v3.1.0), DESeq2 (v1.22.2) microbiome (v1.4.2) and DirichletMultinomial (v1.24.1). For differential abundance testing by DESeq2, the OTU-table was filtered for OTUs present in less than 25% of the samples to minimize zero-variance errors and spurious significance. Permutational multivariate analysis of variance was performed using the adonis function with 999 permutations and Bray-Curtis distances to determine associations between microbiota composition and clinical variables. The Dirichlet Multinomial Mixtures method, using the Laplace equation, was applied for community typing. In this approach samples are clustered based on microbiota profile similarity (11). Kruskal-Wallis followed by post-hoc Dunn's testing was performed to compare Shannon diversity indices between groups.

RESULTS AND DISCUSSION

Before antibiotic treatment, genera *Gardnerella*, *Atopobium*, *Prevotella*, *Lactobacillus* and *Dialister* constituted the core microbiota, and combined accounted for an average relative abundance of 71.9% (**Table 2**), but their abundance could vary greatly between subjects (**Figure 1a**). Two community types could be identified, one driven by *Gardnerella*, *Prevotella*, *Sneathia* and *Atopobium* (community type 1), and one driven by *Lactobacillus*, *Gardnerella* and *Atopobium* (community type 2, **Figure 2a**), suggesting *Lactobacillus*, *Prevotella* and *Sneathia* abundances as discriminative feature of microbiota composition between patients. Bacterial diversity significantly differed between the two community types (**Figure 3a**), with lower diversity in the *Lactobacillus* driven community type. Microbiota composition before treatment was significantly associated with various parameters (**Table 3**), including Nugent score, hormone-related variables (lactation, anticonception use) and BV symptomology (vaginal pH and amine odour).

After treatment, bacterial diversity was decreased (**Figure 3c**) and the core microbiota solely consisted of *Lactobacillus*, constituting an average of 60.8% relative abundance (**Table 2**). Independent of antibiotic type (metronidazole or clindamycin), antibiotic treatment significantly decreased the relative abundance of *Atopobium* (Log2FoldChange = -3.36, padj = 0.0388), while increasing *Lactobacillus* (Log2FoldChange = 4.04, padj = 0.0002). However, *Lactobacillus* remained of low abundance in one-third of the women,

Table 2. Core microbiota before and after antibiotic treatment. Bacterial taxa were considered part of the core microbiota when present in 75% of the samples from the specified group.

	Bacterial genus	Average relative abundance (fraction)
Before treatment	*Gardnerella*	0.294
	Atopobium	0.104
	Prevotella	0.132
	Lactobacillus	0.151
	Dialister	0.038
After treatment	*Lactobacillus*	0.608

Table 3. Clinical variables significantly associated with microbiota composition before and after antibiotic treatment

	Variable	R2	p-value
Before treatment	Nugent score	0.238	0.001
	Anticonception	0.146	0.008
	Lactating	0.091	0.008
	pH > 4.5	0.086	0.012
	Amine odour	0.073	0.028
After treatment	Nugent score	0.499	0.001
	pH > 4.5	0.143	0.006

who's microbiota was of individual-specific composition with high abundance of either *Gardnerella*, *Prevotella*, *Dialister*, *Escherichia-Shigella*, *Atopobium* or *Sneathia* (**Figure 1b**). These microbiota compositions were also reflected by the identification of two community types; one driven by *Lactobacillus*, and the other driven by multiple bacterial taxa (**Figure 2b**), with lower diversity in the *Lactobacillus*-driven community type (community type 1, **Figure 3b**). Vaginal microbiota composition after antibiotic treatment was significantly associated with Nugent score and vaginal pH (**Table 3**).

These findings support the current debate on the definition of a healthy vaginal microbiota (12), since *Lactobacillus* dominance was observed in a large proportion of women with symptoms and the opposite, dominance of anaerobes, was observed in asymptomatic women. So even in a study of small subject size, as herein, heterogeneity of vaginal bacterial communities was apparent. Vaginal health status may be associated with specific *Lactobacillus* species (13), which could not be defined by the method used herein. However, several kinds of microbiota composition existed in asymptomatic women, which has been previously reported (4, 14, 15). Vaginal microbiota composition was consistently associated with Nugent score and vaginal pH. While the Nugent score is considered the golden standard for BV diagnosis, it is rarely used in clinical setting due to resource intensiveness (6).

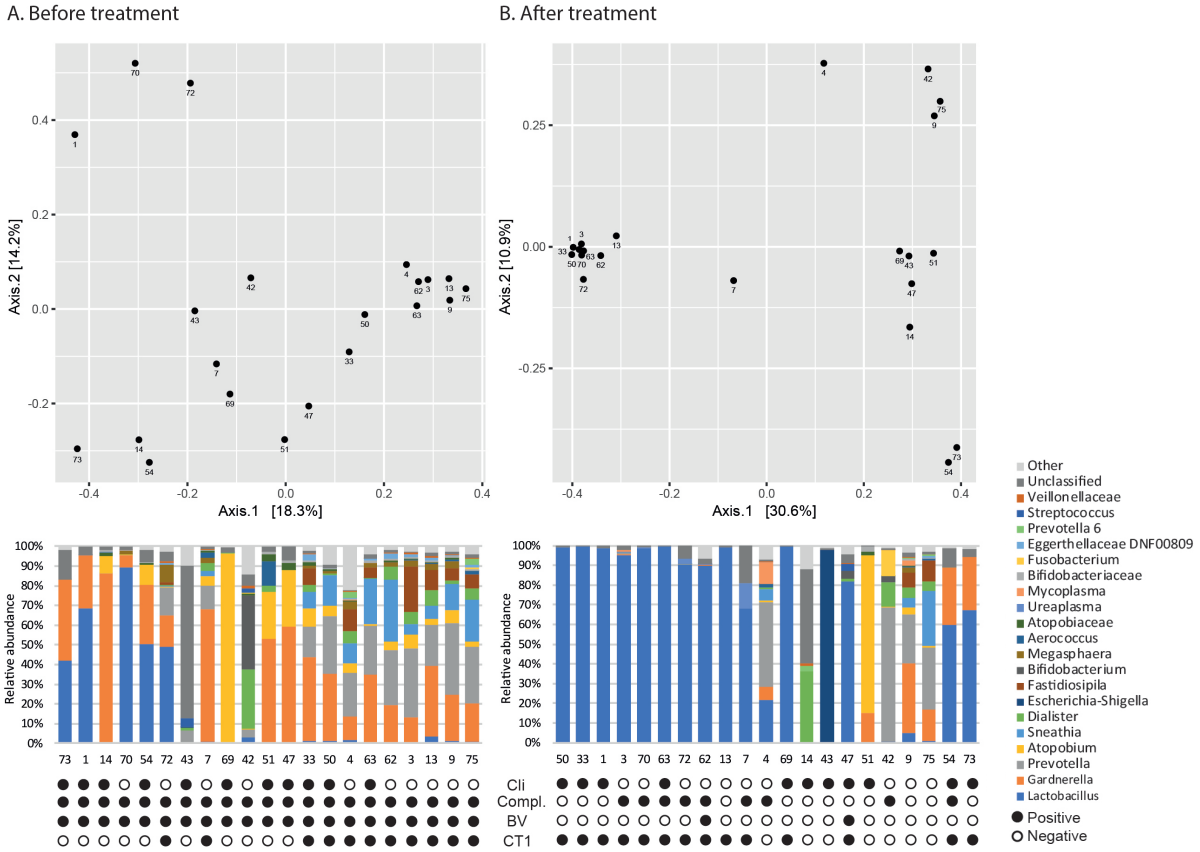

Figure 1. Principal coordinate analysis and taxonomic profiles of the vaginal microbiota before (**a**) and after (**b**) antibiotic treatment. Numbers indicate individual patients. Twenty taxa with highest average relative abundance are shown, abundances of all other taxa are summed and categorised as 'other'. For bargraphs, the subject order is matched to the subject order in the PCoA plots. Cli: clindamycin, Compl.: complaints of abnormal vaginal discharge, CT1: community type one, CT2: community type 2.

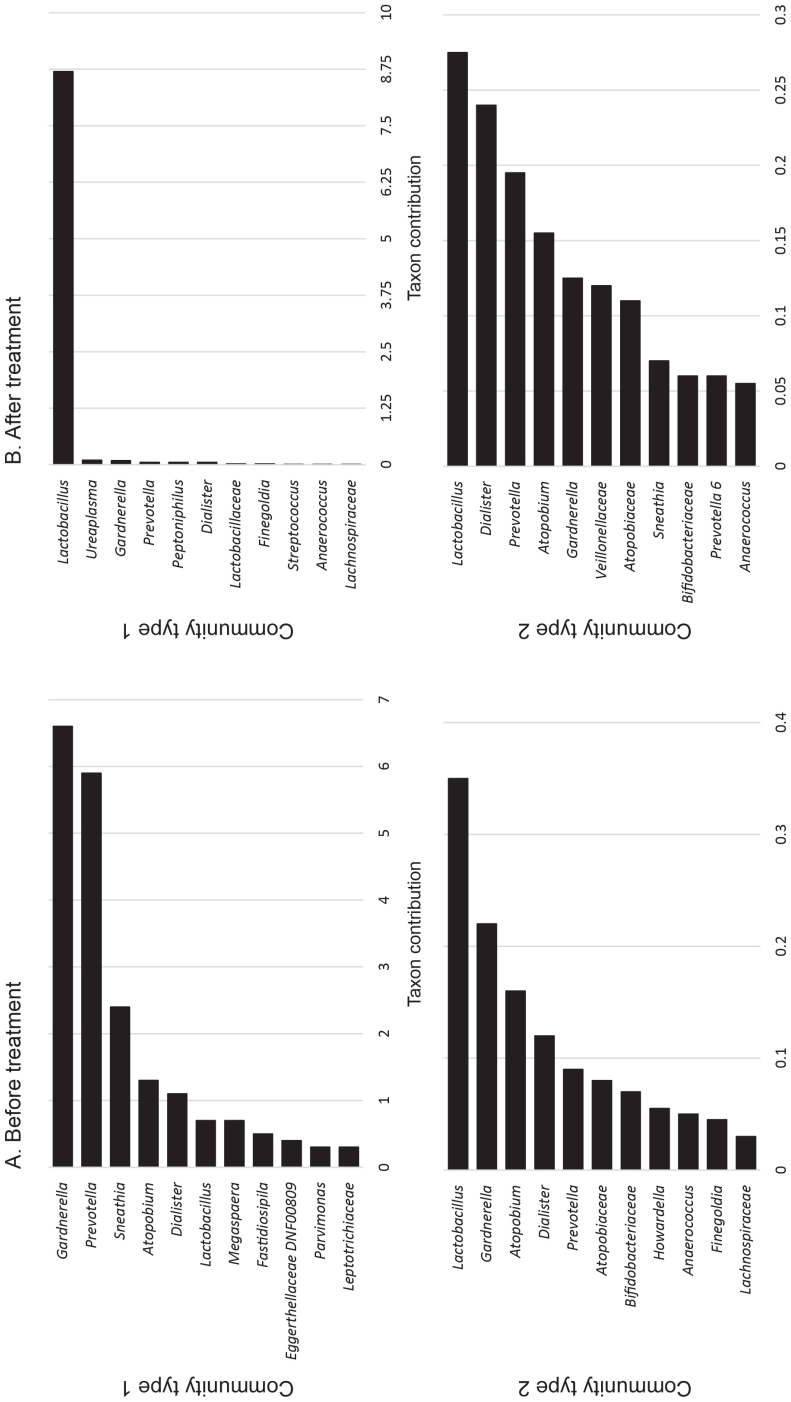

Figure 2. Vaginal microbiota community types before (**a**) and after (**b**) antibiotic treatment. For each community type, the 11 main driving bacterial taxa are shown.

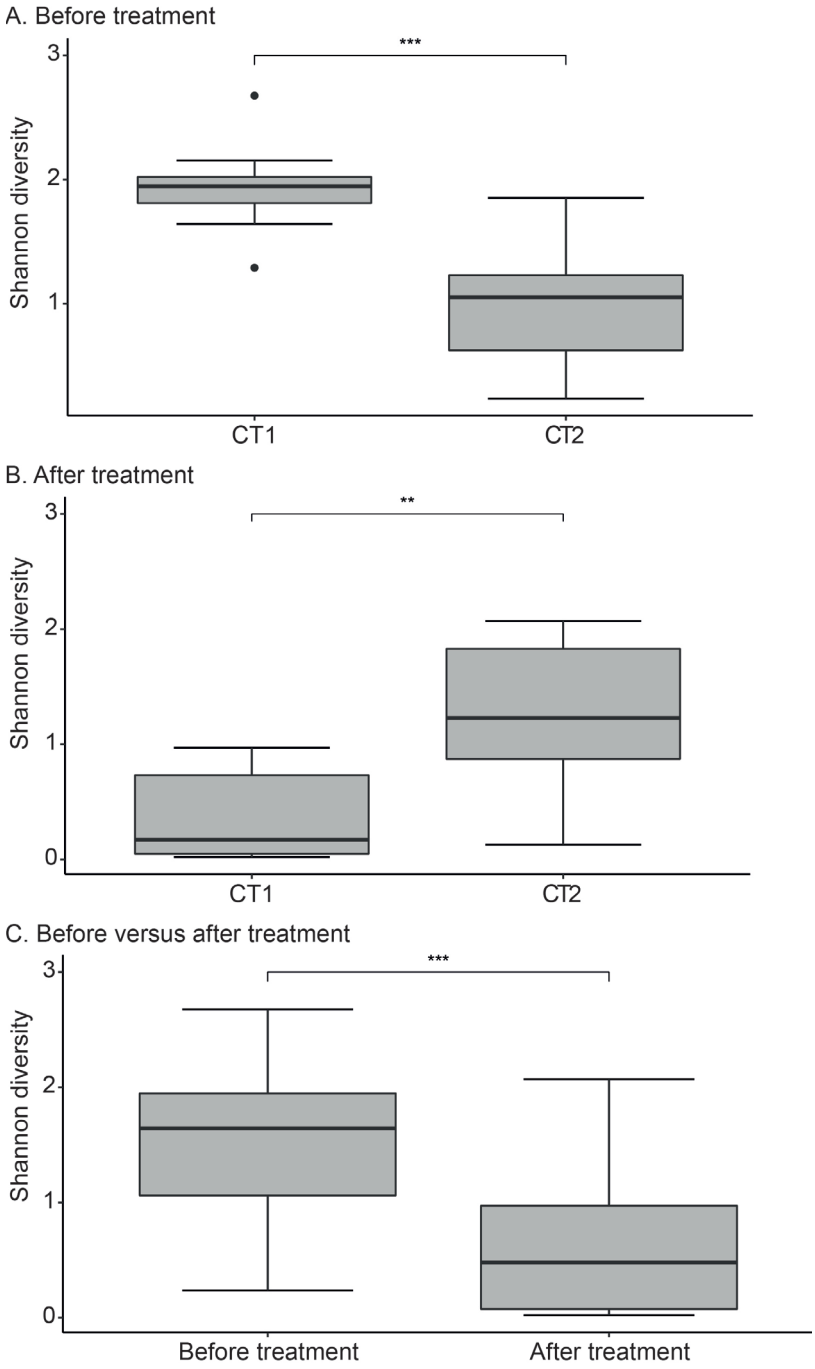

Figure 3. Bacterial diversity of each community type before treatment (**a**) and after treatment (**b**), and of all samples before and after treatment (**c**). Boxplots indicate the median, 25th and 75th percentile and whiskers indicate 1.5* interquartile range. *p < 0.05, **p < 0.01, ***p < 0.001. CT: community type.

Determining vaginal pH is more readily applicable, however, it most certainly simply reflects the abundance of lactic-acid producing bacteria, like *Lactobacillus*. Nowadays, PCR-based laboratory tests would be preferred for confirmation of the diagnosis (16). Except lactation and anticonception use, vaginal microbiota composition was not associated with patient demographics and lifestyle factors, which may be due to the relatively small subject size in combination with uniformity. It has previously been reported that host genetics, ethnicity, hormonal stage (e.g. menstruation cycle, menopause, pregnancy), sexual behaviour and hygiene practices, among others factors, influence vaginal microbiota composition (17-21).

After antibiotic treatment, nine women (43%) reported remaining complaints of abnormal vaginal discharge. Persisting complaints was more prevalent in women receiving metronidazole (70%) than in those receiving clindamycin (18%), which may be a result of differences in antibiotic spectrum and underlying conditions (e.g. pregnancy). To determine the potential influence of the microbiota on clinical outcome, vaginal microbiota composition before and/or after treatment were compared between patients with and without persistent complaints. The vaginal microbiota of women with persisting complaints contained a significantly higher relative abundance of *Ureaplasma* (Log2FoldChange = 8.73, pajd = 0.0008), but persisting complaints could not be associated with microbiota composition before treatment. *Ureaplasma* is a parasitic and saprophytic bacterium belonging to the Mollicutes class and is without cell wall, which results in intrinsic resistance to cell wall-targeting antibiotics like beta-lactam and glycopeptide antibiotics (22). *Ureaplasma* is intrinsically resistant to metronidazole, but usually susceptible to clindamycin (23). While carriage of *Ureaplasma* in urethra, cervix and vagina is common and generally asymptomatic, it has previously been associated with BV recurrence (24). Treatment outcome was not associated with the identified community types after treatment as persistent complaints were reported in 50% (7/14) and 29% (2/7) of women with vaginal microbiota composition belonging to the *Lactobacillus*-driven community type one or multiple species-driven community type two, respectively.

CONCLUSION

In conclusion, defining a (un)healthy vaginal microbiota state remains elusive, which challenges diagnosis and treatment of BV. Abnormal vaginal discharge and itching/irritation is most certainly not attributable to one or more specific bacteria, rather a disruption of the individual-specific mutualistic relationship of bacterial communities. Nevertheless, establishing universal markers for diagnosis and treatment of BV remains relevant. Herein, remaining complaints after treatment was more common in women who received metronidazole and was associated with increased relative abundance of the *Ureaplasma* genus, which may be considered when treatment fails.

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CHAPTER 6

Inter- and intra-patient variability over time of lesional skin microbiota in patients with atopic dermatitis

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ABSTRACT

Atopic dermatitis (AD) is a common chronic, inflammatory skin disorder associated with *Staphylococcus aureus* colonization and reduced microbiota diversity. The current standard for evaluating the effect of treatment on the skin microbiota is by comparing its composition before and after treatment. The aim of the current evaluation was to determine whether limited sampling is sufficient to capture the full extent of variability in the skin microbiota. To analyze inter- and intra-patient variability of the skin microbiota of 20 patients with mild to moderate AD over a period of 42 days, the coefficient of variation (CoV) was calculated for microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration. The inter-patient variability of microbial diversity was high for lesional skin compared to non-lesional skin (CoVs of 35.5-45.9% vs 16.3-28.0%). For the other test results, high CoVs, in the range 45.3-94.1%, were found for lesional skin. furthermore, a wide range of intra-patient variability was observed for lesional skin compared to non-lesional skin (CoVs of 7.1-173% vs 3.5-29.3%). Based on these data, 3 groups with significantly different microbiological phenotypes were defined. In conclusion, lesional skin microbiota is associated with a large inter- and intra-patient variability. A high sample frequency, e.g. once weekly, yields excellent time-dependent insight into the changes of the variable skin microbiota, which can be used to determine the treatment effect on the lesional skin microbiota in clinical trials.

INTRODUCTION

Atopic dermatitis (AD) is a chronic, inflammatory skin disorder characterized by periodic flares of dry, red itchy skin lesions. AD is a very common skin disorder in developed countries with a prevalence of approximately 20% in children and 3% in adults (1). The pathophysiology of AD is complex and still only partially understood. Current evidence strongly points to a disruption of the skin barrier and subsequent immune dysregulation as the primary pathological drivers in AD (2). The microbiome of the skin is important in maintaining immune homeostasis and preventing the skin from being colonized by pathogens, such as *Staphylococcus aureus*. Approximately 90% of the patients suffering from AD are colonized by *S. aureus* and the relative abundance of *S. aureus* increases during an AD flare due to a reduction in the relative abundance of colonizers of the skin (3, 4). *S. aureus* can produce several molecules with potential to cause inflammation and to promote further immune dysregulation (2). Moreover, the increase in relative abundance of *S. aureus* and the reduction of the microbial diversity of the skin seem to be linked to the severity of the disease, promoting the skin microbiota as a potential biomarker in AD (5). Nonetheless, the potential usefulness of this as an AD biomarker has yet to be defined.

Treatments of AD involve emollients and topical anti-inflammatory corticosteroids. There are limitations to the use of steroids, because of possible skin atrophy and systemic side-effects as well as limited patient tolerance after long-term usage. Currently, more specific treatments are being developed (6, 7). The effects of new treatments are increasingly evaluated using subjective clinical AD scores and the microbiota composition of lesional skin before and after treatment (8-11). The design of the majority of these studies includes the collection of a single sample before and after treatment.

However, healthy skin of each human has a specific microbial 'fingerprint', which depends on the physical and chemical features of the skin as well as on host and environmental factors, including colonization at birth, antibiotic exposure, hygiene, lifestyle, and geographic location (12, 13). The level of variation depends on the topographical diversity of the skin as well as on individual factors (14-16). Lesional skin may also be characterized by large inter- and intra-patient variability of the skin microbiota, implying the need for frequent sampling when the effect of a treatment on the lesional skin microbiota is being investigated. However, data of longitudinal studies analyzing the inter- and intra-patient variability of lesional skin microbiota is lacking.

The aim of the current evaluation was to analyze inter- and intra-patient variability of the skin microbiota of patients with AD over time to determine whether limited sampling is sufficient to capture the full extent of variability in the skin microbiota.

MATERIALS AND METHODS

Source of samples and associated data

Microbiological test results of skin swabs, along with selected clinical data from the placebo group of 2 randomized, double-blind, placebo-controlled mono-centre phase 2 clinical trials conducted at the Centre for Human Drug Research (Leiden, The Netherlands) between June 2015 and December 2017, were used in this evaluation. Both clinical trials were approved by the independent Medical Ethics Committee ('Evaluation of Ethics in Biomedical Research', Assen, The Netherlands) and were designed to assess the pharmacodynamics of omiganan in patients with mild to moderate AD. The Declaration of Helsinki was the guiding principle for trial execution. Written informed consent was obtained from all patients.

Data from 250 samples obtained in the initial clinical trial (ClinicalTrials.gov: NCT03091426) were used to determine the variability of the skin microbiota. In this trial, the placebo group (n = 20) consisted of 11 (55%) females and 9 (45%) males with a mean ± standard deviation age of 24 ± 5 years and clinical AD score (objective-scoring atopic dermatitis: oSCORAD) of 21.1 ± 5.6. Briefly, each patient administered the vehicle gel (hydroxyethyl cellulose, sodium benzoate, glycerin, purified water) without the active compound twice daily for 28 consecutive days on all AD lesions. At the start of this treatment period (Day 0), the severity of the lesional skin was assessed clinically. Two skin swabs were collected for bacterial culture and molecular methods using an ESwab and a sterile cotton swab (Puritan, Guilford, ME, USA), respectively. Swabs were dipped in a NaCl-Tween solution, before rubbing the tip of the swab firmly over 4 cm^2 of the target lesion for 5 times. Hereafter the swab material was placed in a vial containing 1 mL NaCl-Tween solution. The skin swabs were obtained from a predefined part of an AD lesion (preferably the antecubital fossa) and from a predefined part of non-lesional skin (preferably the contralateral site). Both clinical assessment and sample collection were repeated each week during a period of 42 days. During the treatment period, patients were allowed to use bland emollients (unguentum leniens) as maintenance therapy. The patients were not allowed to wash the selected sites 6 h prior to the clinical assessment and sample collection and had to avoid prolonged exposure of their involved skin to sunlight during the complete study period. Incomplete datasets or data of samples obtained after concomitant use of corticosteroids were excluded from the analysis.

Data from 76 skin swabs obtained in a separate clinical trial with a comparable study population (ClinicalTrials.gov: NCT02456480) were used for verification purposes. In this trial, the placebo group (n = 12) consisted of 8 (67%) females and 4 (33%) males with an age of 25 ± 11 years and clinical AD score of 19.0 ± 7.4. This clinical trial differed in study design as: (i) the vehicle gel without the active compound was administered once daily on only the predefined AD lesion on the antecubital fossa; (ii) only lesional skin was sampled each week; (iii) clinical assessment of lesional skin was not measured at day 35 and 42; and (iv) bacterial culture was not performed.

Clinical assessment of lesional skin

The severity of the lesional skin was assessed clinically based on the oSCORAD system, calculated as: A/5+7B/2 (17). 'A' in the calculation was defined as the extent of AD, which was assessed as a percentage of each defined body area and reported as the sum of all areas, with a maximum score of 100%. 'B' in the calculation was defined as the severity of 6 specific symptoms of AD (erythema, excoriation, swelling, oozing/crusting, lichenification and dryness), which were scored 0-3 and reported as the sum of all symptoms, with a maximum score of 18. A total score of 0-7.9 was categorized as clear skin, 8.0-23.9 as mild AD, 24.0-37.9 as moderate AD, and 38.0-83.0 as severe AD.

Bacterial culture

Skin swabs were inoculated on blood agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and incubated at 35 °C in a 5% CO_2 incubator for 24 h. Species identification was performed by MALDI-TOF (Bruker Corp., Billerica, MA, USA) and colony-forming units (CFU) were calculated for *S. aureus* after dilution if necessary.

DNA extraction

Each skin swab was diluted by addition of 50 µL 10x phosphate-buffered saline (PBS) to 450 µL swab in NaCL-Tween solution. DNA was extracted and eluted in a final volume of 100 µL with the MagNA Pure 96 instrument using the MagNA Pure 96 DNA and Viral NA Large Volume Kit and the Pathogen universal 500 protocol (Roche Diagnostics, Meylan, France).

Microbiota analysis

Microbiota analysis was performed as described elsewhere (18). Briefly, a fragment of approximately 464 bp of the V3–V4 regions of the 16S ribosomal RNA (rRNA) gene was amplified and sequenced with the MiSeq desktop sequencer (Illumina, San Diego, CA, USA). Sequencing data was processed using the QIIME pipeline and a pre-clustered version of the Augustus 2013 GreenGenes database. High-quality sequences (> 100 bp in length; quality score > 20) were clustered into operational taxonomic units (OTUs) using an open reference-based approach that implements reference-based clustering following by *de novo* clustering at a 97% similarity level. No low abundance filtering was used. For the bar charts, a limited number of genera were selected, representing the microbiota composition of each sample. Only genera with a relative abundance ≥ 1% of the total reads were included. The remaining genera formed the other genera category.

Quantitative real-time PCRs

S. aureus was detected by quantitative real-time PCRs (qPCRs) aimed at the *nuc* gene, using primers and a probe described elsewhere (19). The total bacterial DNA load (16S rRNA gene) was established using a primer set (Fw 5'-CGAAAGCGTGGGGAGCAAA-3', Rv1

5'-CCGTACTCCCCAGGCGG-3' and Rv2 5'-GTCGTACTCCCCAGGCGG-3') based on Bogaert et al. (20) and 20x EVA green (Biotium, Inc., Fremont, CA, USA). Both qPCRs were carried out in a total volume of 10 µL, containing 5 µL (2x) LC480 Probes Master mix (Roche) and 2 µL of extracted DNA. Amplification reactions were performed using a LightCycler 480 II Instrument (Roche) under the following conditions: 5 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 60 °C for 50 s and 72 °C for 1 s (*nuc* gene) or 5 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 60 °C for 15 sec and 72 °C for 1 s (16S rRNA gene). For quantification, a 10-fold dilution series of a plasmid was included in each run and the second derivative analysis method was used for data analysis.

The total load of human DNA (RNaseP gene) was determined using primers and a probe described elsewhere (21). Each qPCR was carried out in a total volume of 25 µL, containing 12.5 µL (2x) IQ Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) and 5 µL of extracted DNA. Amplification reactions were performed using a CFX96 instrument (Bio-Rad Laboratories Inc.) under the following conditions: 3 min at 95 °C followed by 45 cycles of 95 °C for 15 s and 60 °C for 50 s. For quantification, a 10-fold dilution series of MOLT cell line DNA was included in each run. For data analysis, the threshold was set on 850 relative fluorescence units.

Statistical analysis

The statistical software package SPSS was used for statistical analysis. To characterize the microbiota of lesional and non-lesional skin over time, paired sample t-tests and unstructured linear mixed models were performed on the first set of samples. The paired-samples t-test was used to compare microbial diversity (Shannon diversity index) and the relative abundance of *Staphylococcus* spp. of lesional and non-lesional skin at baseline. Unstructured linear mixed model with time as repeated factor was used to compare clinical AD score (oSCORAD), microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration (culture and qPCR) of lesional and non-lesional skin at baseline with data obtained 7, 14, 21, 28, 35 and 42 days later.

To analyze the inter- and intra-patient variability of the lesional and non-lesional skin microbiota, the coefficient of variation (CoV) was calculated for the microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration by dividing the standard deviation by the mean. This was performed for the first and second set of samples. For inter-patient variability, the CoV was calculated per time-point and for intra-patient variability per patient over time. A CoV ≤ 25% has been considered as an acceptable level of variation for analytical methods (22, 23). Clinical data for patient groups were compared using the 1-way analysis of variance (ANOVA) and chi-square tests.

RESULTS

Comparison of lesional and non-lesional skin microbiota

To characterize the microbiota of lesional and non-lesional skin over time, microbiota composition was first compared at baseline. A significant lower microbial diversity of 3.8 ± 1.7 was observed for lesional skin compared to 5.1 ± 1.0 for non-lesional skin (p = 0.011; **Figure 1a**). The lower microbial diversity of the lesional skin was due to the presence of a lower number of OTUs and the relatively high abundance of the genus *Staphylococcus* (**Figure 1b**). Subsequent detection and quantification of *S. aureus* showed a correlation between the relative abundance of the genus *Staphylococcus* and the concentration of *S. aureus* (**Figure 1c, d**). This confirms that the relative abundance of *S. aureus* was higher on lesional skin compared to non-lesional skin as expected.

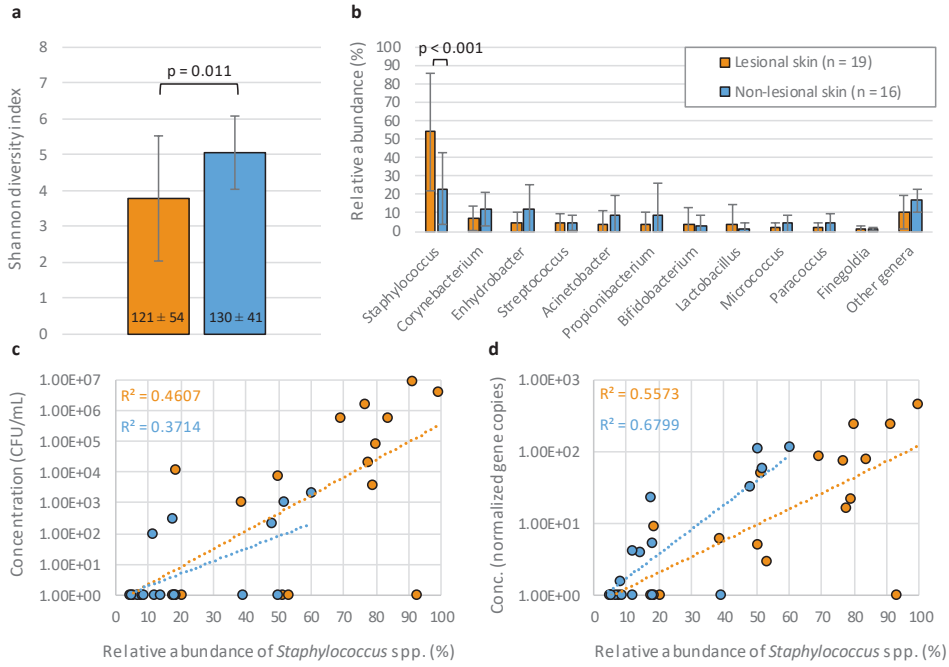

Figure 1. Baseline characteristics of lesional (*orange*) and non-lesional (*blue*) skin microbiota in terms of (**a**) microbial diversity, (**b**) microbiota composition, (**c, d**) *Staphylococcus aureus* concentration based on culture or quantitative real-time PCR (qPCR) in relation to the relative abundance of *Staphylococcus* spp. determined by microbiota analysis. For microbiota diversity, means ± standard deviations of operational taxonomic units are indicated in the bars. The *S. aureus* concentration based on qPCR is normalized by calculating the *nuc* gene copies per 1000 16S rRNA gene copies. All p-values are based on a paired-sampled t-test.

During the following 42 days, the mean clinical AD score of lesional skin was significantly lower ($p \leq 0.036$) compared with the baseline scores (**Figure 2a**). During these days, the mean clinical AD score ranged between 16.1 ± 5.6 and 19.3 ± 4.9, still corresponding with mild to moderate AD. In comparison with the clinical AD score, there was no significant difference in the mean microbial diversity, mean relative abundance of *Staphylococcus* spp. and the mean *S. aureus* concentration determined by qPCR over time (**Figure 2b-e**). The mean microbiota composition of non-lesional skin remained relatively stable over time.

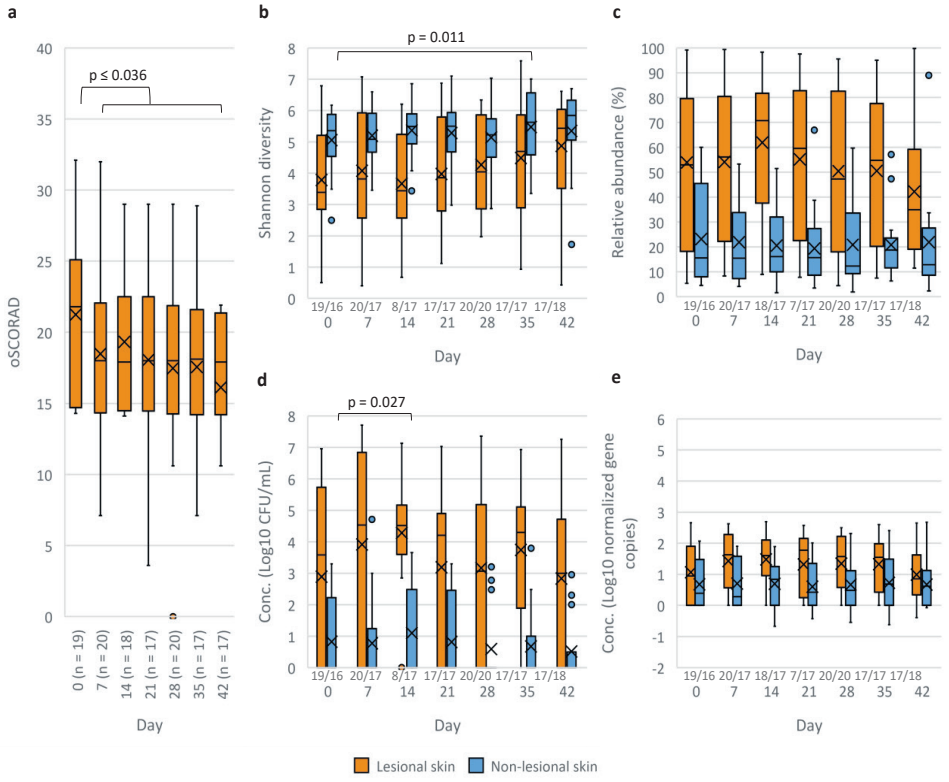

Figure 2. Analysis of lesional (*orange*) and non-lesional (*blue*) skin microbiota during a period of 42 days in terms of (**a**) clinical AD score, (**b**) microbial diversity, (**c**) relative abundance of *Staphylococcus* spp., and (d, e) *Staphylococcus aureus* concentration based on culture or qPCR. The *S. aureus* concentration based on qPCR is normalized by calculating the *nuc* gene copies per 1000 16S rRNA gene copies. Mean values are indicated by crosses and outliers by dots. Number of samples are indicated below the bars. All p-values are based on an unstructured linear mixed model.

Inter-patient variability at each time-point

To quantify the extent of inter-patient variability of the skin microbiota, the CoV was calculated at each time-point for all test results. For lesional skin, high CoVs were observed, in the range 35.5-45.9% for microbial diversity, 46.9-65.2% for relative abundance of *Staphylococcus* spp., and 45.3-94.1% for *S. aureus* concentration. For microbial diversity of non-lesional skin, low CoVs, in the range 16.3-28.0%, were found. These data strongly indicate that there was considerable variation in lesional skin microbiota between patients.

Intra-patient variability over time

To analyze the skin microbiota variability within an individual patient over time, the CoV for microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration was calculated per patient. For all test results of lesional skin, CoVs ranging between 7.1% and 173% were observed. For microbial diversity of non-lesional skin, low CoVs, ranging between 3.5% and 29.3%, were found. These data indicate that there was a wide range of intra-patient variability for lesional skin.

Defining microbiological phenotypes

The patient population could be divided into 3 groups with different microbiological phenotypes, as shown by 3 representative patients in **Figure 3.** The lesional skin microbiota of group I (n = 7; *orange*) and II (n = 8; *blue*) were dominated by *Staphylococcus* spp., resulting in a different profile compared to their non-lesional skin microbiota. These groups differed in variability, as the lesional skin microbiota of group II was relatively unstable (**Supplementary Figure S1**). The lesional skin microbiota of group III (n = 5; *red*) was not dominated by *Staphylococcus* spp. Its composition and variability were similar to their non-lesional skin microbiota. This group had a significantly higher microbial diversity ($p < 0.001$), lower relative abundance of *Staphylococcus* spp. ($p < 0.001$), lower *S. aureus* concentration ($p < 0.001$) and lower clinical AD score ($p = 0.032$) compared with group I and II. There was no significant difference between the 3 groups in age, sex, Fitzpatrick skin type, season of participation, target area for sample collection or total bacterial load.

Confirmation of large inter- and intra-patient variability for lesional skin microbiota

The large inter- and intra-patient variability for lesional skin microbiota was confirmed by data of the second sample set obtained from an independent but comparable study population (**Supplementary Tables S1, S2**). For lesional skin, the CoV for microbial diversity, the relative abundance of *Staphylococcus* spp. and the *S. aureus* concentration at each time-point was between 27.0% and 68.8%. The variability of all test results within an individual patient in time ranged between a CoV of 1.3% and 161.3%. This second sample set also confirmed the existence of 3 different microbiological phenotypes (**Supplementary Table S3, Figure S2**).

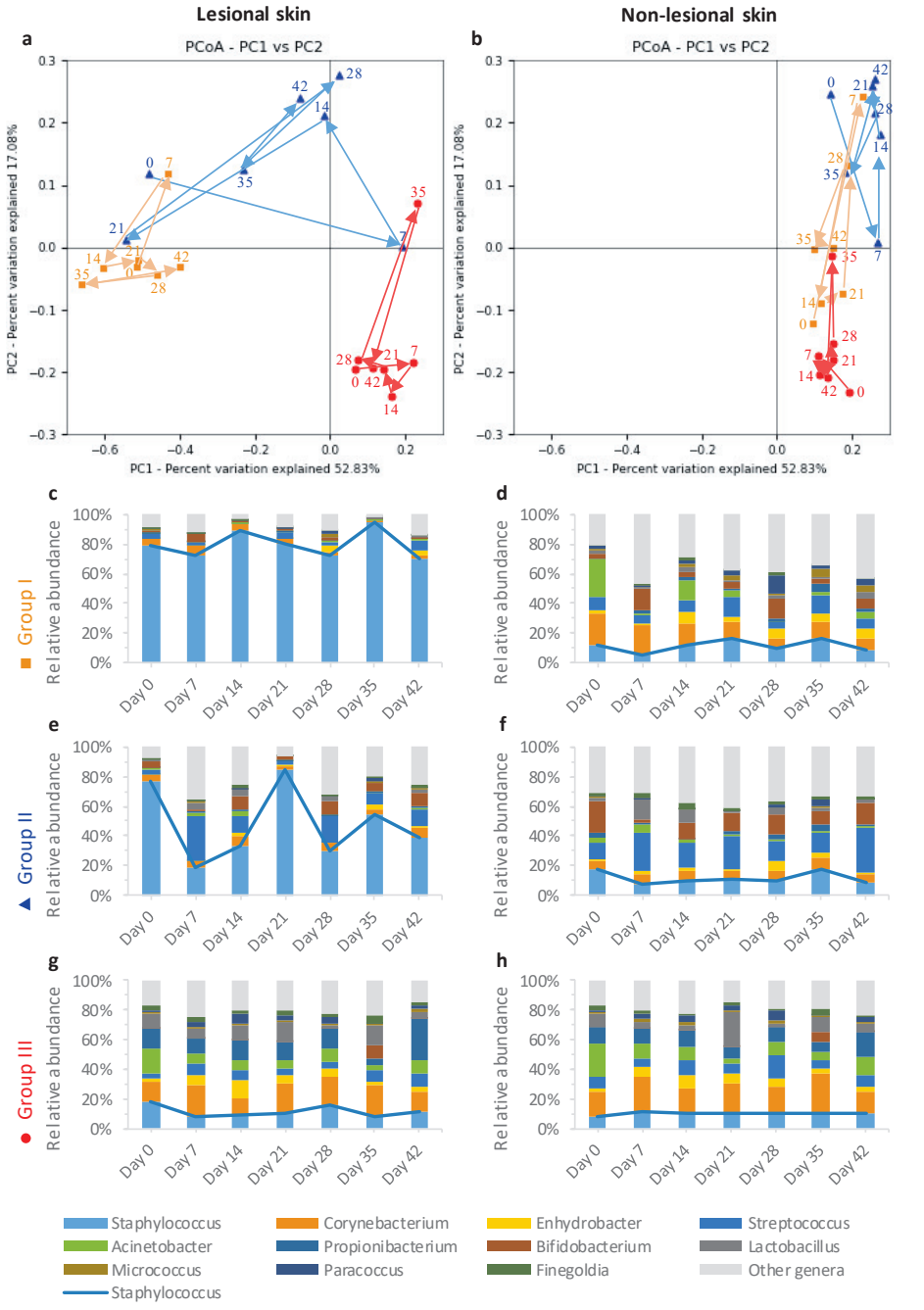

Figure 3. Lesional and non-lesional skin microbiota of 3 selected patients representing 3 groups of patients with different microbiological phenotypes shown in (**a, b**) principal coordinates analysis (PCOA) plots and (**c-h**) bar charts. In the PCOA plots, the arrows combined with the day numbers show how the microbiota composition changed over time.

DISCUSSION

To our knowledge, this is the first longitudinal analysis of inter- and intra-patient variability of skin microbiota of patients with mild to moderate AD. While the sampling method was strictly standardized, large inter- and intra-patient variability for lesional skin microbiota were found. The large inter-patient variability originated from variable *S. aureus* abundance and environmental factors that vary significantly among humans (12, 13). The wide range of intra-patient variability indicated that the skin microbiota of some individuals varied more than others. Based on these data, three patient groups with different microbiological phenotypes were defined. The microbiological phenotype for group I and II can be described as high *Staphylococcal* bioburden, low microbial diversity and either microbiologically stable, or unstable, respectively. The observation that the variability within each of these two groups is consistent within subjects across longitudinal samples, as well as concordant in multiple microbiological assessments, suggests that this difference is not caused by variable sample quality. This difference might be caused by the same unidentified individual (genetic) factors that determine the degree of variability of healthy skin microbiota (15, 16). However, influences of uncontrolled factors (e.g. number of showers, washing with soap, direct UV-exposure) on the stability of the microbiota cannot be excluded. Group III was characterized by a significantly different lesional microbiota compared to group I and II. It could be described as low *Staphylococcal* bioburden and high microbial diversity. The relative lack of dysbiosis was associated with lower clinical AD score.

The data presented in this evaluation suggest that without intervention the individual microbiota composition of the lesional skin can change considerably over a period of 42 days, in particular in patients with a microbiological phenotype of group II. Because the variability over time can be high, single samples collected before and after treatment may not be sufficient to determine the effect of the treatment on an individual's lesional skin microbiota. High sample frequency and statistical analyses methods, which utilize repeated measures across more than one end-of-study time-point, may reduce the effect of the variability in the analyses of clinical trials. The ability to objectively classify subjects to the microbiological phenotypes could be useful in the analyses and interpretation of microbiota data in future clinical trials with larger sample sizes.

The limitation of the presented evaluation is that the sample sets are from patients involved in a clinical trial administering a vehicle gel on AD lesions. Although the vehicle gel did not contain the active compound, this could have had an influence on the lesional skin microbiota as it contains the preservative sodium benzoate. However, this was considered to be minimal because (i) the concentration was far below the minimal inhibitory concentration for *S. aureus* and (ii) the diversity increased under treatment (data not shown). In this evaluation, administration of the vehicle gel had no significant effect on the microbial diversity or relative abundance of *Staphylococcus* spp. However, a significant difference in clinical AS score was

observed after administration of the bland emollient or vehicle gel. Since only one sample for each subject was available prior to initiation of treatment in the clinical trial, we were unable to undertake analyses to evaluate any method to define pre-treatment microbiological stability which could serve as a covariate in statistical analyses or from which to stratify randomization. Another limitation is the small patient group and the omission of including patients of younger age. A larger and more diverse population is required to study the microbiological phenotype classifications and generalize more broadly. Lastly, the limited variation in anatomic target areas and disease states at baseline as inter- and intra-patient variability of lesional skin microbiota might be different for patients e.g. with severe AD located at their dorsal neck.

In conclusion, this evaluation shows that lesional skin microbiota of patient with mild to moderate AD is characterized by large inter- and intra-patient variability, reflecting a highly individual profile. Based on these data, lesional skin microbiota remains a potential target engagement biomarker in AD. A high sample frequency, e.g. once weekly, yields excellent time-dependent insight into the changes of the variable skin microbiota, which can be used to determine the treatment effect on the lesional skin microbiota in clinical trials.

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SUPPLEMENTAL APPENDIX

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PART 3: TRANSLATION OF RESEARCH INTO THE CLINIC

CHAPTER 7

Nasal microbiota dominated by *Moraxella* spp. is associated with respiratory health in the elderly population: a case control study

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ABSTRACT

The elderly (≥65 years) are one of the populations most at risk for respiratory tract infections (RTIs). The aim of this study was to determine whether nasal and/or oropharyngeal microbiota profiles are associated with age and RTIs. Nasal and oropharyngeal swabs of 152 controls and 152 patients with an RTI were included. The latter group consisted of 72 patients with an upper respiratory tract infection (URTI) and 80 with a lower respiratory tract infection (LRTI). Both nasal and oropharyngeal swabs were subjected to microbiota profiling using amplicon sequencing of the 16S rRNA gene. *Moraxella* species were determined using quantitative real-time PCR and culture. Based on the microbiota profiles of the controls and the patients with an RTI, eight nasal and nine oropharyngeal microbiota clusters were defined. Nasal microbiota dominated by either *Moraxella catarrhalis* or *Moraxella nonliquefaciens* was significantly more prevalent in elderly compared to mid-aged adults in the control group ($p = 0.002$). Dominance by *M. catarrhalis/nonliquefaciens* was significantly less prevalent in elderly with an LRTI ($p = 0.001$) compared to controls with similar age. Nasal microbiota dominated by *M. catarrhalis/nonliquefaciens* is associated with respiratory health in the elderly population.

INTRODUCTION

Respiratory tract infections (RTIs) remain one of the leading causes of morbidity and mortality worldwide (1, 2). Whereas upper respiratory tract infections (URTIs) are very common but rarely life threatening, lower respiratory tract infections (LRTIs) are responsible for more severe illnesses, like pneumonia. The populations at risk are the very young (< 5 years) and the elderly (≥ 65 years).

During the first year of life, host and environmental factors, such as genetic predisposition, mode of delivery, infant feeding, exposure to antibiotics, vaccination and geographic location, affect the development of the airway microbiota (3, 4). For the nose, this results gradually in a microbiota profile dominated by *Dolosigranulum, Corynebacterium, Haemophilus, Moraxella, Staphylococcus* and/or *Streptococcus* spp. (5). In the first years of life, microbiota profiles dominated by *Dolosigranulum* and/or *Corynebacterium* spp. are more stable and are positively associated with lower rates of RTIs (6-9). Less stable microbiota profiles characterized by the high abundance of the oral bacteria *Haemophilus* and *Streptococcus* spp. are associated with a higher likelihood of an RTI and their proportion is significantly higher in samples obtained during RTIs when compared to 'healthy' samples (6-10). Furthermore, these microbiota profiles have been associated with an increased risk of recurrent wheeze and asthma in later childhood (9). For microbiota profiles dominated by *Moraxella* spp. variable results have been reported regarding their stability and association with RTIs (6-9). The differences in susceptibility to RTIs likely arise from a complex interplay between mucosa, innate and adaptive immunity, and airway microbiota.

In elderly, the mechanisms of the heightened susceptibility to RTIs are still poorly understood. Immunosenescence, defined as age-related deterioration of both innate and adaptive immunity, seems to impair elderly to elicit effective immune responses against pathogens (11). In addition, immunosenescence might influence the composition of the human microbiota (12, 13). Only few studies have addressed the upper airway microbiota in elderly (14-17), and even less in relation to RTIs (18). The available study typically focusses on the oropharynx for LRTIs, which is suggested to be the main source of microorganisms to the lower airways in adults (19). They observed three microbiota profiles strongly associated with pneumonia and either dominated by *Lactobacillus, Rothia* or *Streptococcus (pseudo) pneumoniae*. In contrast, three other microbiota clusters were correlated with respiratory health and were all characterized by more diverse profiles containing higher abundances of especially *Prevotella, Veillonella* and *Leptotrichia*. However, these microbiota profiles were observed in both in mid-aged adults and elderly. The aim of this study was to determine whether nasal and/or oropharyngeal microbiota profiles are associated with age and RTIs.

MATERIALS AND METHODS

Source of samples

Between Augustus 2012 and Augustus 2014, respiratory swabs were collected from adult patients who were visiting the otorhinolaryngology outpatient clinic or hospitalized at the pulmonary ward of the Reiner de Graaf Gasthuis (Delft, The Netherlands). Two swabs were collected from each patient using sterile flocked swabs (Puritan Medical Products, Maine, USA). One swab was obtained from the head of the concha inferior near the anterior nares and a second swab was obtained from the oropharynx. Each swab was stored in 2 mL STGG (skim milk, tryptone, glucose, glycerol) medium. In total, swabs of 370 patients without clinical symptoms of an RTI and of 211 patients with a suspected URTI or LRTI were collected. For the current analysis, swabs collected from patients with a suspected RTI who received antibiotics in the week before visiting the outpatient clinic and swabs collected from hospitalized patients >1 day after admission were excluded (n = 59 patients), leaving swabs of 152 patients with an RTI for further analysis. Subsequently, swabs of 152 patients without clinical symptoms (i.e. controls) were selected based on sample collection date, age and sex to match the patient group so well as possible. None of them had received antibiotics 1 week prior to sample collection.

Nucleic acid extraction and sequencing

Nucleic acids were extracted from 500 µL STGG medium and eluted in a final volume of 100 µL with the MagNA Pure 96 instrument using the MagNA Pure 96 DNA and Viral NA Large Volume kit and the Pathogen Universal protocol (Roche Diagnostics, Basel, Switzerland). Amplicon sequencing of the 16S ribosomal RNA (rRNA) gene was performed as described elsewhere (20). Briefly, a fragment of ~ 464 bp of the V3-V4 region of the 16S rRNA gene was amplified and sequenced with the MiSeq desktop sequencer (Illumina, San Diego, USA).

Microbiota analysis

Sequencing data was processed following the QIIME1 pipeline. Open reference OTU clustering of high-quality sequences (≥ 100bp in length with a quality score ≥ Q20) was conducted using UCLUST at a 97% similarity level against a pre-clustered version of the Augustus 2013 GreenGenes database. No low abundance filtering was used. See for further details **Supplementary File S1.** Operational taxonomic units (OTUs) with *Alloiococcus* or *Propionibacterium* annotation were renamed. *Dolosigranulum* is known to be misclassified in the GreenGenes database as *Alloiococcus* (21). BLAST search confirmed that the representative sequence matched *Dolosigranulum* in BLAST. *Propionibacterium* spp. have been reclassified to the genus *Cutibacterium* (22).

*Moraxella spe*cies determination

Moraxella species were identified using quantitative real-time PCR (qPCR) and culture. A highly specific qPCR targeting the *copB* gene of *Moraxella catarrhalis* was performed on the isolated DNA of all samples and performed as described elsewhere (20). Culture was performed to determine which *Moraxella* spp. was present in the samples negative for *M. catarrhalis*. For culture, 200-300 µL STGG medium was inoculated on blood agar plates (Becton, Dickinson and Company, New Jersey, USA) and incubated at 35 °C in a 5% CO_2 incubator. Species were identified by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) analyzer with software version 1.6.7.1000 (Bruker corporation, Billerica, USA).

Statistical analysis

For statistical analysis the software package SPSS version 26 was used. Statistically significant differences in variables between the controls and the patients with an RTI was calculated using the Mann-Whitney U and chi-square test for continuous and categorical data, respectively. After the core members of the nasal and oropharyngeal microbiota were determined, hierarchical clusters of microbiota profiles were defined using the free python script 'hierarchical_clustering.py', which was written by Nathan Salomonis of the J. David Gladstone Institutes (San Francisco, CA , USA) and can be found on the following webpage: https://github.com/nsalomonis/altanalyze/blob/master/visualization_scripts/clustering.py. This script uses the Euclidean distance to measure the dissimilarity between each pair of observations. The prevalence of each microbiota cluster per age group was calculated for the controls and the patients with an RTI. Subsequently, the Fisher's Exact test was performed to determine whether microbiota clusters were associated with age and/or RTIs. For the cluster associated with age and RTIs, Fisher's Exact tests were performed to determine whether season of sampling, sex, smoking, young children at home, comorbidities, the use of inhaler or nasal spray were also associated with this cluster in the control group. Furthermore, statistically significant differences in the relative abundance of the genus *Moraxella* between groups was calculated using the Mann-Whitney U test.

RESULTS

Study population

Nasal and oropharyngeal swabs of 152 controls and 152 patients with an RTI were selected (**Table 1**). The 152 controls were visiting the outpatient clinic mainly for an audiogram or hearing complaints (37%), or allergy, skin test or immunotherapy (24%). Of the 152 patients with an RTI, 72 (47%) were suffering from an URTI (i.e. a common cold, sinusitis, tonsillitis or laryngitis). The remaining 80 (53%) patients were hospitalized with a LRTI (i.e. pneumonia,

chronic obstructive pulmonary disease exacerbation, bronchitis or asthma exacerbation), which was diagnosed by the treating physician. Both groups differed significantly in age (p = 0.013).

Table 1. Population characteristics

Group	Controls (n = 152)	Patients with a respiratory tract infection (n = 152)
Age, mean ± SD (range)*	53 ± 19 (18-92)	58 ± 20 (18-89)
Age category, n (%)**		
< 65 years	102 (67)	81 (53)
≥ 65 years	50 (33)	71 (47)
Sex, n (%)		
Female	79 (52)	86 (57)
Male	73 (48)	66 (43)
Season of sampling, n (%)		
Autumn	40 (26)	33 (22)
Winter	47 (31)	59 (39)
Spring	35 (23)	44 (29)
Summer	30 (20)	16 (11)
Reason for visit/hospitalisation, n (%)		
Allergy/skin test/immunotherapy	37 (24)	5 (3)
Audiogram/hearing complaints	56 (37)	7 (5)
Dizzines	9 (6)	0 (0)
Infection	0 (0)	110 (72)
Follow-up	10 (7)	7 (5)
Nose spray	0 (0)	6 (4)
Other; ears[1]	23 (15)	7 (5)
Other; nose[2]	10 (7)	4 (3)
Other; throat[3]	7 (5)	2 (1)
Other; accompaniment	0 (0)	4 (3)
Upper airway infection, n (%)	Not applicable	47 (31)
Common cold		4 (3)
Laryngitis		14 (9)
Sinusitis		7 (5)
Tonsillitis		
Lower airway infection, n (%)		2 (1)
Asthma exacerbation		3 (2)
Bronchitis		24 (16)
COPD exacerbation		51 (34)
Pneumonia		

COPD: chronic obstructive pulmonary disease; SD: standard deviation.

Statistically significant differences in variables between both groups was calculated using the Mann-Whitney U and chi-square test for continuous and categorical data, respectively. *p = 0.013. **p = 0.014.

[1]Other; ears included cleaning of ears and inserting grommets.

[2]Other; nose included septum deviation, frequent nosebleeds and choanal polyp.

[3]Other; throat included complaints of long-lasting cough or difficult swallowing movement.

Determination of the core microbiota of the nasal passages and oropharynx

To determine whether the nasal and/or oropharyngeal microbiota were associated with age and RTIs, first core microbiota profiles were defined using amplicon sequencing of the 16S rRNA gene. A mean of 77,414 reads per swab were obtained with sequencing, which resulted in a mean OTU of 50 for the nasal swabs and 83 for the oropharyngeal swabs.

In the nasal passages of the 152 controls and 152 patients with an RTI, the 10 most abundant genera/families were *Corynebacterium* (mean relative abundance of 28%), *Staphylococcus* (24%), *Moraxella* (12%), *Dolosigranulum* (7%), *Streptococcus* (5%), *Haemophilus* (3%), *Peptoniphilus* (3%), *Cutibacterium* (2%), *Anaerococcus* (2%), and Enterobacteriaceae (2%). Together these bacteria account for 88% of the classified sequences. Interestingly, three microbiota profiles were dominated (i.e. ≥ 50% relative abundance) by one of the less abundant genera *Pseudomonas* and *Neisseria*.

In the oropharynx, *Prevotella* (mean relative abundance of 26%), *Veillonella* (16%), *Streptococcus* (11%), *Neisseria* (7%), *Fusobacterium* (6%), *Leptotrichia* (5%), *Haemophilus* (5%), *Rothia* (3%), *Porphyromonas* (3%), and *ActinoBacillus* (2%) were the 10 most abundant genera, accounting for 84% of the classified sequences. Four microbiota profiles were dominated by *Lactobacillus* or *Staphylococcus*.

These bacteria are the core members of the nasal and oropharyngeal microbiota of the controls and patients with an RTI. Separate analyses for both patient groups resulted in comparable core members (**Supplementary Table S2**).

Microbiota clustering analysis based on nasal or oropharyngeal core members

To define clusters of microbiota profiles, hierarchical clustering was performed based on the nasal or oropharyngeal core members (**Supplementary Figure S3**). For the nasal passages, eight microbiota clusters were defined (**Supplementary Table S4a**). Cluster I was characterized by a relatively high abundance of *Haemophilus*, *Neisseria* or *Streptococcus* (Hae/Nei/Str), II by *Moraxella* (Mor), III by *Staphylococcus* and *Corynebacterium* (Sta, Cor), IV by *Corynebacterium* and *Dolosigranulum* (Cor, Dol), V by *Corynebacterium* (Cor), VI by *Staphylococcus* (Sta), VII by *Moraxella* and *Corynebacterium* (Mor, Cor), and VIII by *Dolosigranulum, Haemophilus, Cutibacterium,* Enterobacteriaceae or *Streptococcus* (Dol/Hae/Cut/Ent/Str). These microbiota clusters had a mean Shannon diversity index ranging between 2.18 and 4.50.

For the oropharynx, nine microbiota clusters were defined of which cluster I was characterized by a relatively high abundance of *Prevotella* and *Fusobacterium* (Pre, Fus), II/III by *Prevotella* and *Veillonella* (Pre, Vei), IV by *Prevotella* (Pre), V by *ActinoBacillus, Haemophilus, Staphylococcus, Rothia* or *Neisseria* (Act/Hae/Sta/Rot/Nei), VI by *Streptococcus* and *Veillonella* (Str, Vei), VII by *Lactobacillus* (Lac), VIII by *Streptococcus* and *Rothia* (Str, Rot), and IX by *Streptococcus, Neisseria, ActinoBacillus, Lactobacillus* or *Staphylococcus* (Str/Nei/Act/Lac/Sta) (**Supplementary Table S4b**). Compared to the nasal microbiota clusters, the

microbiota profiles within the oropharyngeal microbiota clusters were more variable which was illustrated by the mean Shannon diversity index ranging between 3.13 and 6.45. There was no correlation between the nasal and oropharyngeal microbiota clusters.

Nasal and oropharyngeal microbiota clusters related to higher age in the control group

After clusters of nasal and oropharyngeal microbiota profiles were defined, their prevalence in the control group was calculated per age group (adults aged ≥ 65 years or < 65 years). Of the eight nasal microbiota clusters, three microbiota clusters were associated with age (**Figure 1a**). Cluster II (Mor) and IV (Cor, Dol) were significantly more prevalent in adults aged ≥ 65 years compared to adults aged < 65 years ($p \leq 0.019$), whereas cluster III (Sta, Cor) was significantly less prevalent in adults aged ≥ 65 years ($p = 0.037$). Of the nine oropharyngeal microbiota clusters, cluster VI (Str, Vei) was significantly more prevalent in adults aged ≥ 65 years ($p = 0.015$; **Figure 1b**). These data showed that specific microbiota profiles of both the nasal passages and oropharynx are associated with higher age.

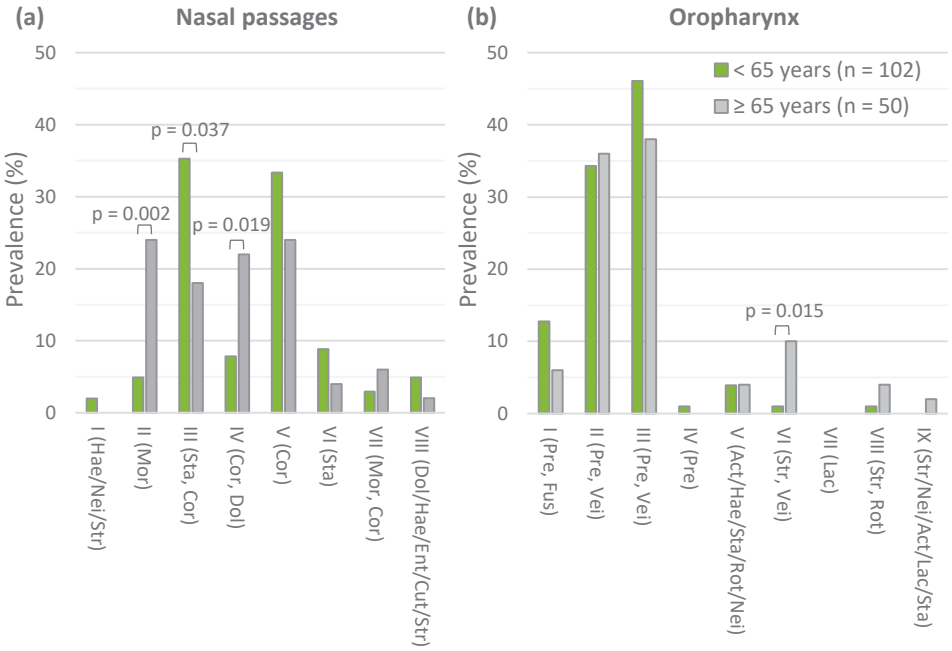

Figure 1. Prevalence of microbiota clusters among the controls aged < 65 and ≥ 65 years. (**a**) nasal microbiota clusters. (**b**) oropharyngeal microbiota clusters. Act: *ActinoBacillus*; Cor: *Corynebacterium*; Cut: *Cutibacterium*; Dol: *Dolosigranulum*; Ent: Enterobacteriaceae; Fus: *Fusobacterium*; Hae: *Haemophilus*; Lac: *Lactobacillus*; Mor: *Moraxella*; Nei: *Neisseria*; Pre: *Prevotella*; Rot: *Rothia*; Sta: *Staphylococcus*; Str: *Streptococcus*; Vei: *Veillonella*. Genera separated from each other by a comma are both represented in a relatively high abundance in each microbiota profile of the relevant cluster. Genera separated from each other by a slash indicates that one of these genera is present in a relatively high abundance. All p-values are based on Fisher's Exact test. Correction for multiple testing was not performed.

Nasal and oropharyngeal microbiota clusters related to higher age and RTIs

Subsequently, the prevalence of the nasal and oropharyngeal microbiota clusters of the patients with any RTI (**Figure 2a, b**), URTI (**Figure 2c, d**) or LRTI (**Figure 2e, f**) were compared to the control group. Nasal microbiota cluster II (Mor) was strongly associated with higher age and LRTI as it was significantly less prevalent in patients with a LRTI who passed the age of 65 years compared to controls with similar age ($p = 0.001$).

Nasal microbiota cluster VI (Sta; $p = 0.039$), oropharyngeal microbiota cluster III (Pre, Vei; $p = 0.037$) and oropharyngeal microbiota cluster V (Act/Hae/Sta/Rot/Nei; $p = 0.042$) were moderately associated with LRTIs in patients aged < 65 years. Interestingly, oropharyngeal microbiota cluster VII (Lac) was only present in patients with a LRTI who passed the age of 65 years. These data indicate that both the nasal and oropharyngeal microbiota differed between the controls and patients with a LRTIs.

Nasal microbiota cluster II dominated by *Moraxella* spp.

Of all identified microbiota clusters, nasal cluster II (Mor) was of most interest since it was associated with higher age and less prevalent in elderly with a LRTI compared to the healthy elderly population. This finding was strengthened by the significant difference in mean abundance of *Moraxella* spp. between the age groups of the controls ($p = 0.003$) and between the controls and patients with a LRTI who passed the age of 65 years ($p = 0.008$; **Table 2**). In the control group, no association with season of sampling, sex, smoking, young children at home, comorbidities, the use of inhaler or nasal spray was found.

To determine whether *M. catarrhalis* was representing nasal cluster II (Mor), a qPCR was performed. Of all 29 nasal swabs, five (18%) were positive for *M. catarrhalis*. Culture data suggested that the remaining 24 (82%) of the swabs within nasal cluster II (Mor) were represented by *M. nonliquefaciens*.

Table 2. Mean relative abundance of *Moraxella* spp. per population group

Population group	All ages		< 65 years		≥ 65 years	
	Mean ± SD (%)	Range (%)	Mean ± SD (%)	Range (%)	Mean ± SD (%)	Range (%)
Controls and patients	12 ± 26	0-100	9 ± 23	0-100	15 ± 29	0-100
Controls	13 ± 28	0-100	7 ± 20*	0-99	25 ± 37 */**/***	0-100
Patients	10 ± 24	0-100	12 ± 27	0-100	8 ± 20**	0-81
Patients with URTI	15 ± 29	0-100	14 ± 29	0-100	15 ± 30	0-81
Patients with LRTI	6 ± 18	0-100	8 ± 23	0-100	6 ± 15***	0-67

LRTI: lower respiratory tract infection; SD: standard deviation; URTI: upper respiratory tract infection.
Statistically significant differences between groups was calculated using the Mann-Whitney U test. *p = 0.003. **p = 0.018. ***p = 0.008.

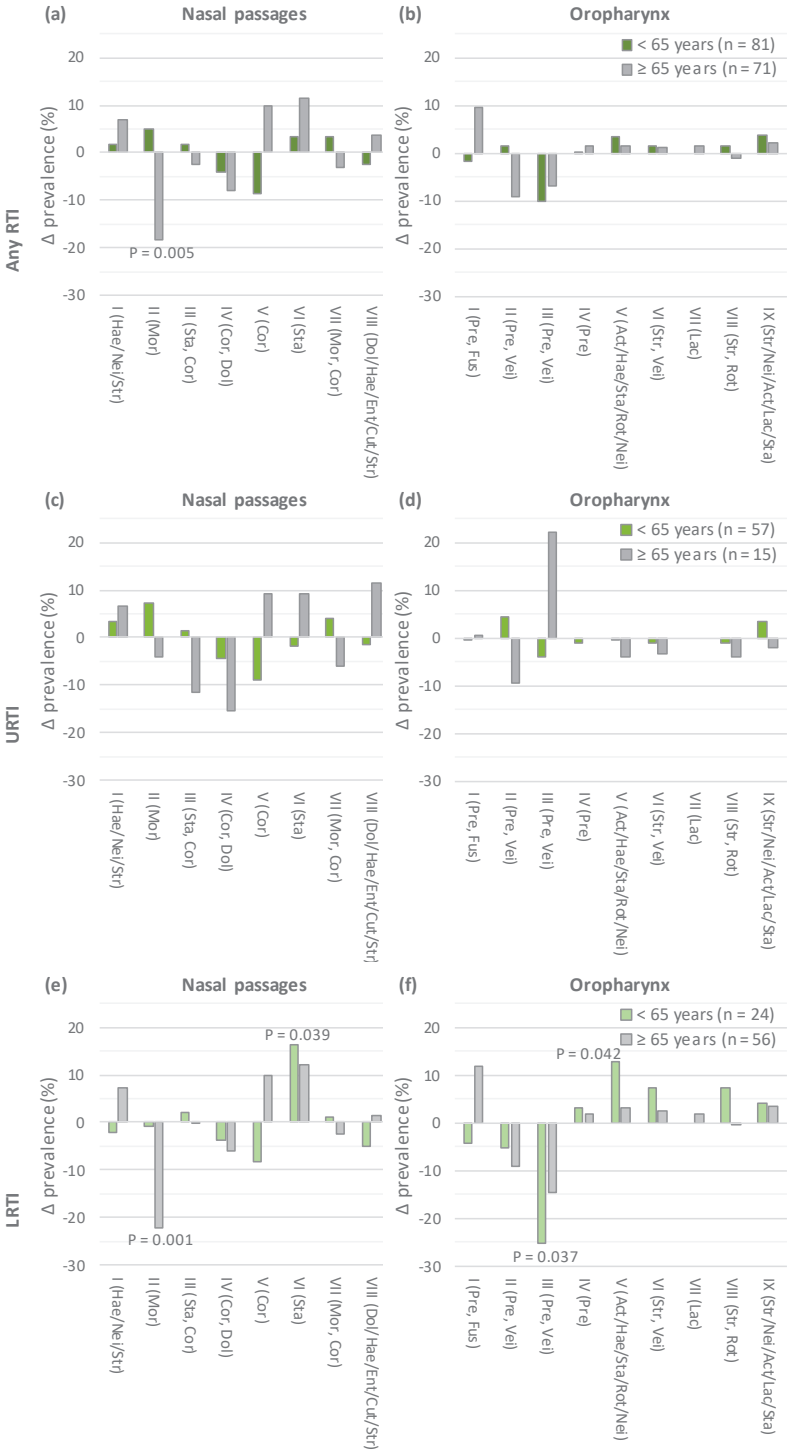
Any RTI
URTI
LRTI
(a)
Nasal passages
(b)
Oropharynx
< 65 years (n = 81)
≥ 65 years (n = 71)
(c)
Nasal passages
(d)
Oropharynx
< 65 years (n = 57)
≥ 65 years (n = 15)
(e)
Nasal passages
(f)
Oropharynx
< 65 years (n = 24)
≥ 65 years (n = 56)
Δ prevalence (%)
20
10
0
-10
-20
-30
P = 0.005
P = 0.039
P = 0.001
P = 0.042
P = 0.037
I (Hae/Nei/Str)
II (Mor)
III (Sta, Cor)
IV (Cor, Dol)
V (Cor)
VI (Sta)
VII (Mor, Cor)
VIII (Dol/Hae/Ent/Cut/Str)
I (Pre, Fus)
II (Pre, Vei)
III (Pre, Vei)
IV (Pre)
V (Act/Hae/Sta/Rot/Nei)
VI (Str, Vei)
VII (Lac)
VIII (Str, Rot)
IX (Str/Nei/Act/Lac/Sta)

Figure 2. Comparison of prevalence of microbiota clusters between controls and patients per age group. (**a**) nasal and (**b**) oropharyngeal microbiota clusters of controls and patients with any respiratory tract infection (RTI). (**c**) nasal and (**d**) oropharyngeal microbiota clusters of controls and patients with an upper respiratory tract infection (URTI). (**e**) nasal and (**f**) oropharyngeal microbiota clusters of controls and patients with a lower respiratory tract infection (LRTI). Act: *ActinoBacillus*; Cor: *Corynebacterium*; Cut: *Cutibacterium*; Dol: *Dolosigranulum*; Ent: Enterobacteriaceae; Fus: *Fusobacterium*; Hae: *Haemophilus*; Lac: *Lactobacillus*; Mor: *Moraxella*; Nei: *Neisseria*; Pre: *Prevotella*; Rot: *Rothia*; Sta: *Staphylococcus*; Str: *Streptococcus*; Vei: *Veillonella*. Genera separated from each other by a comma are both represented in a relatively high abundance in each microbiota profile of the relevant cluster. Genera separated from each other by a slash indicates that one of these genera is present in a relatively high abundance. All P-values are based on Fisher's Exact test. Correction for multiple testing was not performed.

DISCUSSION

To the best of our knowledge, this is the largest study on the nasal and oropharyngeal microbiota and its relation to both URTIs and LRTIs in elderly. Based on the microbiota profiles of the controls and the patients with an RTI, we defined eight nasal and nine oropharyngeal microbiota clusters. One of the nasal microbiota clusters was strongly associated with age and RTIs.

The results of this study showed that nasal cluster II dominated by *M. catarrhalis/nonliquefaciens*, was significantly more prevalent in the healthy elderly population compared to the healthy mid-aged adults. Interestingly, *M. catarrhalis/nonliquefaciens* was significantly less prevalent in elderly with a LRTI compared to the healthy elderly population, suggesting an association between *M. catarrhalis/nonliquefaciens* and respiratory health in elderly. Previous reports have shown that *Moraxella* spp. become predominant community members over time in most young children (5-9). Their microbiota as well as their immune system are in development, whereas both innate and adaptive immunity seem to deteriorate in elderly (11). In essence, it might tolerate the same bacterial species. This might explain the significantly higher prevalence of *M. catarrhalis/nonliquefaciens* in the healthy elderly population. However, conflicting results have been reported regarding the role of *Moraxella* spp. in the pathogenesis of RTIs in young children. Some studies found that profiles dominated by *M. catarrhalis/nonliquefaciens* was associated with respiratory health (5-8), while others reported that *Moraxella* spp. were associated with high susceptibility to LRTIs (9). Since *M. catarrhalis* has been considered as being a pathogen for certain disease entities (e.g. COPD exacerbation and otitis media), it is most likely that *M. nonliquefaciens* is actually associated with respiratory health.

Nasal and oropharyngeal microbiota clusters moderately associated with LRTIs were characterized by a relatively high abundance of a potential pathogen, such as *Staphylococcus, ActinoBacillus, Haemophilus,* and *Rothia* spp. The difference in prevalence compared to the healthy population was observed in both age groups but was only significantly different in the mid-age adults. This means that no microbiota cluster was defined that could elucidate

why elderly are more susceptibility to LRTIs. However, the data does indicate that both the nasal and oropharyngeal microbiota have impact on lower airway health in adults while it is generally assumed that only the oropharynx is involved in the pathogenesis of LRTI (22).

A cross-sectional study of Steenhuijsen Piters and colleagues revealed 11 (sub)clusters of oropharyngeal microbiota profiles (18). Three clusters were associated with pneumonia which were characterized by a relative high abundance of *S. (pseudo)pneumoniae, Rothia* spp. or *Lactobacillus* spp. In contrast, three other microbiota clusters were correlated with respiratory health and contained high abundances of *Prevotella, Veillonella* and *Leptotrichia*. In our study, *Streptococcus, Rothia* and *Lactobacillus* dominated only a limited number of oropharyngeal microbiota profiles. Notably, the oropharyngeal microbiota cluster characterized by a relatively high abundance of *Lactobacillus* was only covered by patients with a LRTI. Furthermore, we observed a moderate association between an oropharyngeal microbiota cluster with high abundances of *Prevotella* and *Veillonella* and respiratory health in mid-aged adults.

URTIs are mainly caused by viruses and previous reports have shown that *Streptococcus* and *Haemophilus* spp. are associated with viral infections (23-26). The interactions between viruses and the airway microbiota may affect the course of the disease and subsequent respiratory health (27). In our study, nasal microbiota clusters characterized by a high abundance of *Streptococcus* or *Haemophilus* spp. were associated with the presence of respiratory viruses in patients with a URTI (data not shown). However, no significant difference in prevalence was observed compared to the healthy population.

A limitation of this study is that the data was collected at one timepoint. Longitudinal and more comprehensive data regarding microbiota composition and function as well as immunogenic status is required in order to elucidate the mechanism of the heightened susceptibility to RTIs in elderly. Longitudinal data is also required to confirm that nasal microbiota has impact on the lower airway health in adults. Stronger correlations might have been found when data was used from a matched case-control study, controls were healthy relatives of the patients, only patients with a confirmed pneumonia were included, nasopharynx was sampled and when specimens were collected during hospital admission, reducing antibiotic usage prior to specimen collection. Lastly, sputum collection in case of a LRTI would have been valuable to identify the causative pathogen (20).

CONCLUSIONS

We showed that nasal microbiota dominated by *M. catarrhalis/nonliquefaciens* is associated with respiratory health in the elderly population. Further research is required to determine which species is associated with respiratory health and whether it is a positive association. In case of a positive association, efforts should be made to uphold these bacteria to promote respiratory health in the elderly population.

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SUPPLEMENTAL APPENDIX

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CHAPTER 8

Balanopreputial sac and urine microbiota in patients with male genital lichen sclerosus

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ABSTRACT

Male genital lichen sclerosus (MGLSc) is a chronic inflammatory scarring dermatosis associated with penile carcinoma. The prepuce is pivotal in its aetiology. Other proposed aetiological factors are the subject of dispute, and include occluded urinary exposure, autoimmunity, immune-dysregulation and infectious agents. The objective of this study was to determine whether the bacterial microbiota of the balanopreputial sac and urine are associated with MGLSc. 20 uncircumcised patients with MGLSc and 20 healthy uncircumcised males were enrolled in a prospective case-control study. Balanopreputial swabs and urine specimens were subjected to 16S rRNA gene amplicon sequencing. Microbiota analysis indicated differences between the groups. In the balanopreputial sac, the median relative abundance of *Finegoldia* spp. was lower (9% [range 0% – 60%] in MGLSc patients than in controls (28% [range 0% – 62%]). Conversely, the median relative abundance of *Fusobacterium* spp. was higher in MGLSc patients (4% [range 0% – 41%]) than in controls (0% [range 0% – 28%]). In the urine, the median relative abundance of *Finegoldia* spp. was comparable between groups, whereas that of *Fusobacterium* spp. was higher in MGLSc patients (0% [range 0% – 18%] vs 0% [range 0% – 5%]). There was a strong association between the microbiota composition of the balanopreputial sac and urine in MGLSc. In conclusion, dysbiosis could be involved in the aetiopathogenesis of MGLSc. Further studies are required to confirm the association suggested herein and determine its nature.

INTRODUCTION

Lichen sclerosus (LSc) is a chronic lichenoid inflammatory fibrosing disorder with a predilection for genital skin (1, 2). Genital (G)LSc can cause substantial dermatological, sexual and urological morbidity and predisposes to intraepithelial neoplasia and squamous cell carcinoma (SCC) (1, 2). The aetiology is disputed (1, 2). Claims have been advanced for autoimmunity, immunodysregulation, and infective agents (1-4). The obligate role of the foreskin in male (M)GLSc is acknowledged; MGLSc is exceptionally rare in those circumcised at birth and circumcision is usually curative. The exact role of the foreskin is unclear, although occlusion and koebnerisation appear relevant. Unlike in women, male GLSc does not affect perianal or perineal skin; the exception to this rule is the observation of peri-urethrostomy LSc in males, indicating a link with urinary exposure. Furthermore, MGLSc is associated with high rates of post-micturition 'micro-incontinence' (2, 5). Together, these observations have led to the hypothesis that MGLSc arises from occluded exposure of a susceptible epithelium to urine. However, to date, no specific constituent or property of urine nor specific susceptibility factor has been identified (2, 6, 7).

The defining characteristics of the LSc are inflammation, sclerosis and neoplasia, but the pathogenesis remains poorly defined at a molecular level. It is plausible that a unified pathogenic pathway could account for each of these characteristics. In recent years, evidence has emerged i) that variations in the composition of the human microbiota may contribute to the development of many hitherto pathogenically unexplained chronic inflammatory diseases (8-10) and ii) that commensal bacteria can beneficially regulate host immunity, decreasing the risk of infection-induced autoimmune diseases and/or inflammation (11). Conversely, maladaptive alterations in microbiota composition (dysbiosis) may lead to perturbed immune homeostasis and disease (11). In addition, dysbiosis is implicated in fibrotic disease (12) and carcinogenesis (8, 13).

The ecosystem of the skin is divided into multiple anatomical 'niches' each with a site-specific microbiota (14). Various inflammatory dermatoses manifest preferentially at certain sites; for example, acne predominantly affects the face, chest and back (14), while LSc usually affects the genitalia. It is conceivable that the genital predilection of LSc could reflect site-specific dysbiosis. Previous studies of the penile microbiota indicate that circumcision dramatically changes its composition, with significant decreases in the abundance of anaerobic bacteria (15). Anaerobic bacteria in the coronal sulcus of uncircumcised men correlate with the presence of specific inflammatory cytokines (16), several of which are elevated in LSc (17, 18).

The microbiota composition of the balanopreputial sac has not previously been investigated in MGLSc. Dysbiosis may account for unresolved questions about the exact nature of the relationship between urine and epithelial susceptibility in MGLSc, and the pathways from lichenoid inflammation to fibrosis and carcinogenesis. Hence this analysis of the microbiota of the balanopreputial sac and urine in MGLSc.

MATERIALS AND METHODS

Study design

Ethical approval for this prospective case-control study was obtained through the NHS Health Research Authority Research Ethics Service (RES); London – Riverside Research Ethics Committee references 07/H0706/62, amendment number 4.

Twenty uncircumcised adult males with untreated, clinically pathognomonic MGLSc were recruited from the Male Genital Dermatoses Clinic at our institution. The clinical diagnosis of MGLSc was based on a comprehensive history and examination by highly experienced clinicians. Controls were recruited from general dermatology clinics and the Male Genital Dermatoses Clinic. They were uncircumcised, with clinically healthy balanopreputial tissue. No participant was immunocompromised.

From each participant, a swab of the balanopreputial sac (glans and inner prepuce) and a first void urine sample was collected. The swab was obtained by rubbing a sterile cotton swab (Puritan, Guilford, ME, USA) soaked in 1 mL 0.9% NaCl Tween Solution firmly five times against across the balanopreputial skin. First-void urine was collected using a Colli-Pee (Novosanis, Wijnegem, Belgium). All samples were stored at -20 °C until DNA extraction.

DNA extraction

DNA was extracted from 200 µL samples and eluted in a final volume of 50 µL with the MagNA pure 96 instruments using the MagNA pure 96 DNA and Viral NA small volume kit and the pathogen universal protocol (Roche Diagnostics, Basel, Switzerland).

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed as described elsewhere (19). Briefly, the total bacterial load of the samples was established by targeting the 16S rRNA gene. Amplification reactions were performed using a LightCycler 480 Instrument (Roche Diagnostics) under the following conditions: 5 min at 95 °C followed by 45 cycles of 95 °C for 10 sec, 60 °C for 15 sec and 72 °C for 1 sec, and a final step of 10 sec at 40 °C. For quantification, a 10-fold dilution series of a plasmid was included in each run and the second derivative analysis method was used for data analysis. A concentration of ≥ 5.00E+02 copies/µL was considered positive.

Amplicon sequencing of the 16S rRNA gene

Amplicon sequencing of the 16S rRNA gene to determine the microbiota composition was performed as described elsewhere (19). Briefly, a fragment of approximately 464 bp of the V3-V4 region of the 16S rRNA gene was amplified using the universal primers described by Klindworth et al. (20). PCR products with a positive agarose gel result were further processed and subsequently sequenced with the MiSeq desktop sequencer (Illumina, San Diego, CA, USA).

Sequencing data was processed following the standard QIIME pipeline. High quality sequences (length > 100 bp, quality score > 20) were clustered at a 97% similarity level using a pre-clustered version of the Augustus 2013 Greengenes database. Low abundance OTUs (a fraction < 0.005 of all sequences) were removed. A sample was considered positive for a specific genus when more than 1% of the sequences were assigned to that genus.

Statistical analysis

The software package SPSS version 26 was used for statistical analysis. For bacterial load, alpha diversity and relative abundance data, the Mann Whitney U test was employed. For analysis of association between urinary and balanopreputial data, Fisher's exact test was performed, only for patients with available microbiota data for both balanopreputial sac and urine.

RESULTS

Population characteristics

Twenty uncircumcised patients with MGLSc and 20 healthy uncircumcised males were enrolled in this study. The MGLSc patients had a median age of 37 years (range 26-73). The control group had a median age of 42 years (range 19-63).

Bacterial load

To determine whether the bacterial load in the balanopreputial sac and urine differed between MGLSc patients and controls, a qPCR targeting the 16S rRNA gene was performed (**Supplementary Figure S1, Table S2**). For the balanopreputial sac, the median bacterial load was 8.20 (range 5.34 – 9.44) log gene copies/mL for the MGLSc patients and 7.88 (range 5.57 – 9.24) log 16S rRNA gene copies/mL for the controls. The median bacterial load of the urine was 4.23 (range 0 – 6.18) log gene copies/mL for the MGLSc patients and 4.26 (range 0.98 – 7.19) log 16S rRNA gene copies/mL for the controls. No significant difference in bacterial load between both groups in either the balanopreputial sac ($p = 0.758$) or urine ($p = 0.235$) was observed.

Microbiota analysis

To determine the bacterial microbiota composition of the balanopreputial sac and urine, amplicon sequencing of the 16S rRNA gene was performed. For seven MGLSc patients and five controls, the bacterial load of the urine specimen was too low for microbiota analysis. For the remaining specimens, the alpha-diversity, beta-diversity and microbiota profiles were analysed.

The median alpha-diversity was 2.61 and 2.17 for the balanopreputial sac of MGLSc patients and the controls, respectively (**Figure 1**). For urine, it was 2.38 and 2.33 for MGLSc patients and the controls, respectively. No significant difference was observed between the MGLSc patients and the controls in either the balanopreputial sac ($p = 0.201$) or urine ($p = 1.000$).

Beta-diversity analysis of the balanopreputial sac showed a relatively high variation in microbiota composition between the MGLSc patients and between the controls (**Figure 2a**). The microbiota composition of the balanopreputial sac differed between the MGLSc patients and the controls, whereas that of the urine was more comparable between the groups (**Figure 2b**).

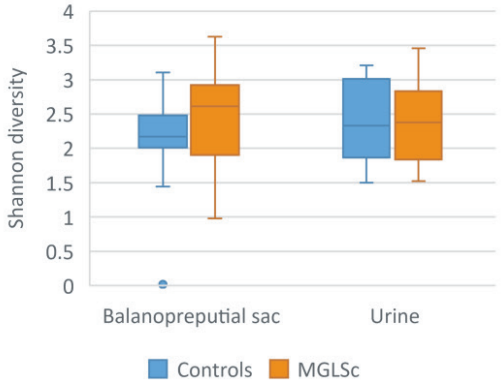

Figure 1. Alpha diversity of swabs of balanopreputial sac and urine of controls and MGLSc patients

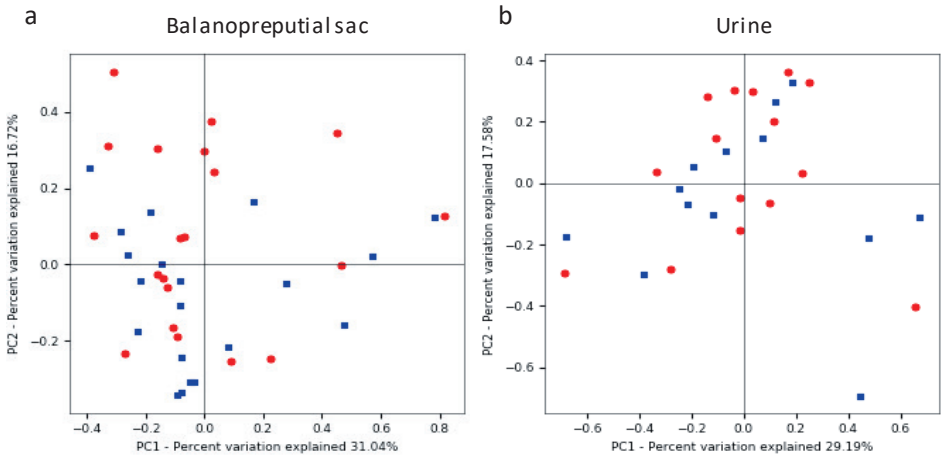

Figure 2. Unifrag weighted PCOA plots of (**a**) balanopreputial sac and (**b**) urine of healthy controls and MGLSc patients. The blue squares are the healthy controls and the red dots are the MGLSc patients

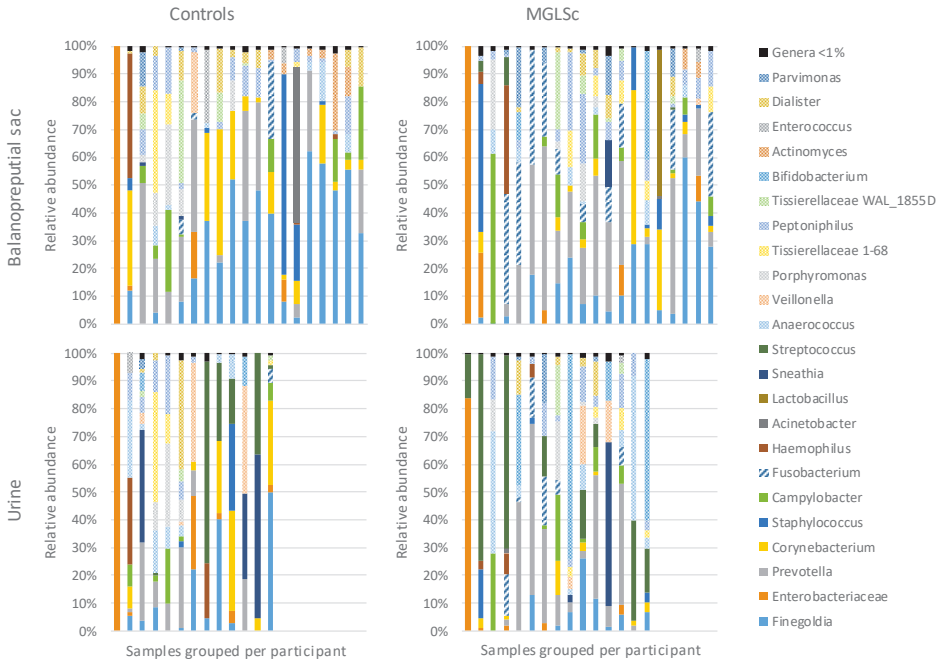

Figure 3. Microbiota composition of the balanopreputial sac and urine in healthy controls and MGLSc at genus level

Analysis of the microbiota profiles of both balanopreputial sac and urine indicated differences between the groups (**Figure 3, Supplementary Table S3, S4**). In the balanopreputial sac, the genus *Finegoldia* was present in 17 (85%) of both the MGLSc patients and controls, but the median relative abundance of 9% (range 0% – 60%) in MGLSc patients was lower than in controls (28%, range 0% – 62%) (**Supplementary Table S5**). Furthermore, the presence and/or relative abundance of the genera *Fusobacterium* and *Prevotella* was higher in patients with MGLSc. *Fusobacterium* spp. were present in 10 (50%) of the patients, while it was detected in 3 (15%) of the controls; its median relative abundance was higher in MGLSc patients (4% [range 0% – 41%] vs 0% [range 0% – 28%]). *Prevotella* was present in 15 (75%) of the patients with MGLSc and 11 (55%) of the controls; its median relative abundance was higher in MGLSc patients (20% [range 0% – 59%] than in controls (4% [range 0% – 51%]).

The microbiota profiles of the urine specimens differed between the participants of both groups and from the skin microbiota (**Supplementary Table S6**). However, there was an association between the presence of several genera, including *Fusobacterium*, in the balanopreputial sac and urine in patients with MGLSc (**Supplementary Table S7**). These data suggest an association between the microbiota of the urine and that of the balanopreputial sac.

DISCUSSION

To our knowledge this represents the first combined investigation of the composition of the microbiota of both the balanopreputial sac and urine in MGLSc. The presented data suggests that dysbiosis of the balanopreputial sac microbiota could be involved in MGLSc. The relative abundance of *Finegoldia* spp. was decreased in patients with MGLSc while the relative abundance of *Fusobacterium* spp. was higher in patients with MGLSc. *Fusobacterium* spp. exhibit properties and associations that could be relevant to the defining characteristics of MGLSc.

Recent advances in culture-independent microbial detection technology have shed light on the important role of the genus *Fusobacterium*, in particular *F. nucleatum*, in inflammatory diseases such as periodonitis and inflammatory bowel disease (21). *In vitro* and *In vivo* studies of the specific pro-inflammatory properties of *Fusobacterium* spp. indicate potential relevance in LSc; *F. nucleatum* upregulates the anti-microbial peptides hBD-2 and S100A7 that are also upregulated in LSc (22-25). *F. nucleatum* also upregulates several cytokines, including IL-1, IL-4, IL-6, CCL5, CXCL10, CXCL11 and TNF-α that are upregulated in LSc (17, 18, 21-23, 26-28).

LSc is a fibrosing disease (1). The cause of fibrogenesis remains unexplained by previously proposed aetiological factors, and the mechanism leading to aberrant fibrogenesis in LSc has not been the focus of specific investigation. Studies in other fibrotic diseases such as systemic sclerosis and idiopathic pulmonary fibrosis implicate perturbations in the Wnt/β-catenin (29, 30) and mitogen activated protein kinase (MAPK) signalling pathways, including the subfamilies JNK and p38 MAPK (31, 32). These pathways are involved in regulation of extracellular matrix deposition and degradation and collagen gene expression, and induction of these pathways can cause fibrosis (31-35). Whether dysbiosis could play a role in aberrant fibrogenesis in LSc remains unknown. Several *Fusobacterium* spp. have the capacity to activate the Wnt/β-catenin and MAPK signaling pathways, including JNK and p38 MAPK (36, 37). *In vitro* studies of keratinocyte lines have shown that *Fusobacterium* spp., upregulate secretion of matrix metalloproteinases (MMPs), including MMP-1, MMP-2 and MMP-9, in keratinocytes (23, 26, 38) that are also upregulated in LSc (18, 39, 40). The pathway of fibrogenesis in LSc, and any involvement of the microbiota therein, require further investigation.

MGLSc and human papillomavirus represent the two main risk factors for penile carcinoma (41, 42). However, little is known of the molecular mechanisms involved in progression from MGLSc to penile carcinoma, and the role of dysbiosis has not been investigated in this context. The role of dysbiosis in carcinogenesis has demonstrated in other cancers, including oral squamous cell carcinoma and colorectal carcinoma; studies have highlighted the important role of *F. nucleatum* in particular (13, 37, 43-47). Studies in murine models and *In vitro* studies indicate that *F. nucleatum*-mediated carcinogenesis

is effected via upregulation of Wnt/β-catenin signaling and STAT3 signaling (37, 41, 48). Upregulation of Wnt/β-catenin signaling is observed in penile carcinoma (49). These observations suggest that *Fusobacterium* spp. deserve specific focus in future studies of the link between MGLSc and penile carcinoma.

Circumcision has a dramatic impact on the composition of the penile microbiota, significantly reducing the abundance of anaerobes, particularly *Fusobacterium* spp. (15, 50). The changes in microbiota composition following circumcision could explain the curative and preventative effect of circumcision in MGLSc.

Involvement of the urethra in MGLSc appears to support the aetiological role of urine (1), however the specific property of urine has not been identified (1, 6); the strong association identified between the balanopreputial sac and urine microbiota composition may account for the link. While this study represents the only study to date of both the balanopreputial microbiota and urinary microbiota, Cohen et al. investigated the urinary microbiota of men with urethral MGLSc and demonstrated a unique microbiota profile in MGLSc compared with controls, with enrichment of the orders Bacillales, Bacteroidales and Pasteurellales (51). They did not report any differences in the relative abundance of the order Fusobacteriales. The variable findings may relate to methodology in specimen collection; while Cohen et al. analysed mid-stream urine, first void urine was analysed herein, and was selected on the basis that urethral involvement in MGLSc typically develops distally and progresses proximally (52). Notably, *Borrelia* spp., previously proposed as an aetiological factor in MGLSc (2), was not identified in any specimen in this study.

The notion that dysbiosis may be involved in the aetiopathogenesis of MGLSc is a novel one. This study, with a low number of subjects, is essentially pilot in nature; as such, no firm conclusions can be drawn. Other limitations include the lack of longitudinal data preclude determination of whether balanopreputial dysbiosis represents the cause or consequence of the disease. The diagnosis of MGLSc was based on clinical features and histopathology was not sought to confirm the diagnosis. There has been historical discussion regarding the role of biopsy in the diagnosis of LSc; the literature indicates that histology may be non-specific and falsely negative, and reliance on histology can lead to delayed diagnosis and progression of the disease (53, 54). For these reasons, histopathology was not sought in this study. Identification of bacteria at the genus level also represents a limitation, since virulence factor expression can vary between species or even strains (8). Finally, the clinical phenotype arising from dysbiosis is likely to depend upon factors such as genetic predisposition (8), but host factors were not analysed herein. Further studies with increased numbers of participants are required to confirm the results and investigate the molecular role, if any, that dysbiosis may play in MGLSc.

CONCLUSION

This study suggests that dysbiosis of the balanopreputial sac microbiota may play a role in the aetiopathogenesis of MGLSc. Further studies are required to determine if dysbiosis could form the 'missing link' between occlusion, urinary exposure, epithelial susceptibility, inflammation, fibrosis and carcinogenesis.

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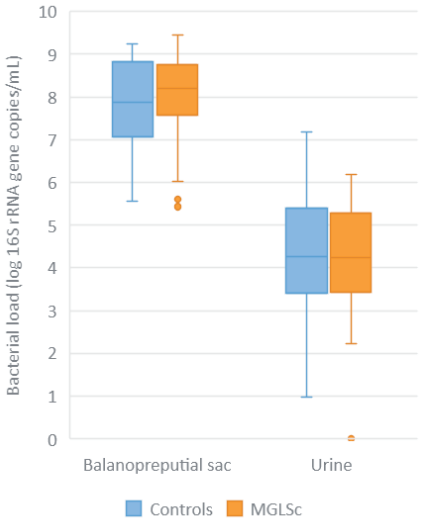
SUPPLEMENTAL APPENDIX

Supplementary Figure S1. Bacterial load of balanopreputial sac and urine in healthy controls and MGLSc patients



Supplementary Table S2. (**a**) Bacterial load of balanopreputial swabs; (**b**) Bacterial load of first catch urine

a.

Controls		**MGLSc**	
SampleID	16S copies/mL	Sample ID	16S copies/mL
1	5.91E+08	5	3.78E+07
2	8.60E+08	6	1.48E+08
4	4.95E+06	8	2.70E+05
7	1.70E+09	10	5.94E+08
9	4.11E+07	14	1.05E+06
11	6.36E+07	15	4.05E+07
12	1.09E+09	18	5.54E+08
13	6.54E+05	19	4.10E+08
16	1.81E+08	20	1.60E+08
17	3.73E+05	21	6.53E+07
22	2.28E+07	23	1.41E+07
26	8.76E+06	24	5.58E+08
27	9.04E+07	29	6.35E+08
28	2.03E+08	30	1.07E+08
32	6.31E+07	31	1.09E+09
33	1.63E+07	35	1.60E+08
34	1.01E+07	36	2.26E+08
37	3.03E+08	41	2.78E+09
38	1.74E+09	43	2.53E+08
42	6.98E+08	44	3.98E+05

b.

Controls		**MGLSc**	
SampleID	16S copies/mL	Sample ID	16S copies/mL
1	1.38E+05	5	1.71E+02
2	1.85E+04	6	4.58E+03
4	1.06E+03	8	5.24E+02
7	2.89E+06	10	1.18E+04
9	1.30E+04	14	2.48E+04
11	6.70E+04	15	3.55E+03
12	1.39E+06	18	1.51E+06
13	3.05E+05	19	4.49E+04
16	5.50E+02	20	4.99E+02
17	9.61E+00	21	1.25E+05
22	6.00E+03	23	0.00E+00
26	2.29E+03	24	2.54E+03
27	1.25E+06	29	4.05E+05
28	1.79E+04	30	1.07E+04
32	6.06E+03	31	2.16E+05
33	1.83E+04	35	2.49E+05
34	9.30E+01	36	1.44E+05
37	3.59E+03	41	1.39E+06
38	7.04E+04	43	1.34E+05
42	1.55E+07	44	1.15E+04

Supplementary Table S3. Number of bacterial genera found in each group

	Balanopreputial sac	Urine	Total	Present in both samples
Control	20	20	22	18
MGLSc	23	21	23	21
Total	23	21	23	21
Present in both groups	21	20	22	18

Supplementary Table S4. Bacterial genera found in each of the four groups (balanopreputial sac in healthy controls, balanopreputial sac in MGLSc, urine in healthy controls, urine in MGLSc)

Genus	Controls		MGLSc	
	Balanopreputial sac	Urine	Balanopreputial sac	Urine
Acinetobacter	+	-	+	+
Actinomyces	+	-	+	-
Anaerococcus	+	+	+	+
Bifidobacterium	-	+	+	+
Campylobacter	+	+	+	+
Corynebacterium	+	+	+	+
Dialister	+	+	+	+
Enterobacteriaceae	+	+	+	+
Enterococcus	+	+	+	+
Finegoldia	+	+	+	+
Fusobacterium	+	+	+	+
Haemophilus	+	+	+	+
Lactobacillus	-	-	+	-
Parvimonas	+	+	+	+
Peptoniphilus	+	+	+	+
Porphyromonas	+	+	+	+
Prevotella	+	+	+	+
Sneathia	+	+	+	+
Staphylococcus	+	+	+	+
Streptococcus	-	+	+	+
Tissierellaceae 1-68	+	+	+	+
Tissierellaceae WAL_1855D	+	+	+	+
Veillonella	+	+	+	+

Supplementary Table S5. Balanopreputial sac: prevalences and median relative abundances of bacteria in healthy controls and MGLSc patients

Genus	Prevalence n (%)			Median relative abundance % (Range)		
	Control (n=20)	MGLSc (n=20)	*p*	Control	Urine	*p*
Acinetobacter	1 (5)	1 (5)	1.000	0 (0-56)	0 (0-1)	0.989
Actinomyces	6 (30)	2 (10)	0.235	0 (0-28)	0 (0-8)	0.314
Anaerococcus	6 (30)	7 (35)	1.000	0 (0-14)	0 (0-9)	0.738
Bifidobacterium	0 (0)	2 (10)	0.487	0 (0-0)	0 (0-39)	0.602
Campylobacter	8 (40)	9 (45)	1.000	0 (0-30)	0 (0-61)	0.799
Corynebacterium	13 (65)	11 (55)	0.748	3 (0-45)	2 (0-55)	0.314
Dialister	10 (50)	7 (35)	0.523	0 (0-16)	0 (0-8)	0.289
Enterobacteriaceae	4 (20)	5 (25)	1.000	0 (0-100)	0 (0-100)	0.758
Enterococcus	2 (10)	1 (5)	1.000	0 (0-27)	0 (0-5)	0.779
Finegoldia	17 (85)	17 (85)	1.000	28 (0-62)	9 (0-60)	0.086
Fusobacterium	**3 (15)**	**10 (50)**	**0.041**	**0 (0-28)**	**4 (0-41)**	**0.033**
Haemophilus	3 (15)	2 (10)	1.000	0 (0-45)	0 (0-39)	0.820
Lactobacillus	0 (0)	1 (5)	1.000	0 (0-0)	0 (0-52)	0.799
Parvimonas	2 (10)	6 (30)	0.235	0 (0-12)	0 (0-22)	0.265
Peptoniphilus	15 (75)	15 (75)	1.000	2 (0-17)	3 (0-28)	0.968
Porphyromonas	5 (25)	4 (20)	1.000	0 (0-29)	0 (0-25)	0.779
Prevotella	11 (55)	15 (75)	0.320	4 (0-51)	20 (0-59)	0.265
Sneathia	2 (10)	1 (5)	1.000	0 (0-2)	0 (0-17)	0.820
Staphylococcus	5 (25)	7 (35)	0.731	0 (0-72)	0 (0-54)	0.640
Streptococcus	0 (0)	3 (15)	0.231	0 (0-0)	0 (0-10)	0.429
Tissierellaceae 1-68	3 (15)	5 (25)	0.695	0 (0-37)	0 (0-13)	0.620
Tissierellaceae WAL_1855D	4 (20	5 (25)	1.000	0 (0-37)	0 (0-28)	0.841
Veillonella	1 (5)	1 (5)	1.000	0 (0-22)	0 (0-2)	0.989

Supplementary Table S6. Urine - prevalences and median relative abundances of bacteria of healthy controls and MGLSc patients

Genus	Prevalence n (%)			Median relative abundance % (Range)		
	Control (n=13)	MGLSc (n=15)	*p*	Control	Urine	*p*
Acinetobacter	0 (0)	1 (7)	1.000	0 (0-0)	0 (0-2)	0.786
Actinomyces	0 (0)	0 (0)	-	0 (0-0)	0 (0-0)	1.000
Anaerococcus	7 (54)	7 (47)	1.000	2 (0-28)	0 (0-52)	0.856
Bifidobacterium	2 (15)	4 (27)	0.655	0 (0-11)	0 (0-74)	0.496
Campylobacter	5 (38)	6 (40)	1.000	0 (0-20)	0 (0-28)	0.856
Corynebacterium	6 (46)	7 (47)	1.000	0 (0-36)	0 (0-12)	0.496
Dialister	3 (23)	4 (27)	1.000	0 (0-39)	0 (0-12)	0.856
Enterobacteriaceae	6 (46)	5 (33)	0.700	0 (0-100)	0 (0-84)	0.496
Enterococcus	1 (8)	1 (7)	1.000	0 (0-7)	0 (0-2)	0.964
Finegoldia	9 (69)	8 (53)	0.460	4 (0-50)	1 (0-26)	0.467
Fusobacterium	1 (8)	6 (40)	0.084	0 (0-5)	0 (0-18)	0.130
Haemophilus	2 (15)	3 (20)	1.000	0 (0-31)	0 (0-7)	0.964
Lactobacillus	0 (0)	0 (0)	-	0 (0-0)	0 (0-0)	1.000
Parvimonas	1 (8)	3 (20)	0.600	0 (0-3)	0 (0-17)	0.618
Peptoniphilus	6 (46)	10 (67)	0.445	0 (0-21)	3 (0-16)	0.440
Porphyromonas	3 (23)	4 (27)	1.000	0 (0-30)	0 (0-21)	0.964
Prevotella	7 (54)	11 (73)	0.433	1 (0-29)	3 (0-61)	0.274
Sneathia	3 (23)	2 (13)	0.639	0 (0-59)	0 (0-59)	0.650
Staphylococcus	2 (15)	3 (20)	1.000	0 (0-31)	0 (0-18)	0.856
Streptococcus	6 (46)	8 (53)	1.000	0 (0-73)	8 (0-74)	0.650
Tissierellaceae 1-68	3 (23)	4 (27)	1.000	0 (0-40)	0 (0-8)	0.964
Tissierellaceae WAL_1855D	3 (23)	2 (13)	0.639	0 (0-4)	0 (0-18)	0.751
Veillonella	4 (31)	3 (20)	0.670	0 (0-39)	0 (0-21)	0.650

Supplementary Table S7. Association between the balanopreputial sac and urine bacterial microbiota

Genus	Control (n = 13)			MGLSc (n = 15)		
	Urine+ when Balanopreputial sac-, ratio (%)	Urine+ when Balanopreputial sac+, ratio (%)	*p*	Urine+ when Balanopreputial sac-, ratio (%)	Urine+ when Balanopreputial sac+, ratio (%)	*p*
Acinetobacter	0/13 (0)	0/0 (0)	-	1/15 (7)	0/0 (0)	-
Actinomyces	0/12 (0)	0/1 (0)	-	0/15 (0)	0/0 (0)	-
Anaerococcus	5/10 (50)	2/3 (67)	1.000	**2/9 (22)**	**5/6 (83)**	**0.041**
Bifidobacterium	2/13 (15)	0/0 (0)	-	2/13 (15)	2/2 (100)	0.057
Campylobacter	**1/8 (13)**	**4/5 (80)**	**0.032**	**0/9 (0)**	**6/6 (100)**	**<0.001**
Corynebacterium	1/6 (17)	5/7 (71)	0.103	**1/8 (13)**	**6/7 (86)**	**0.010**
Dialister	0/5 (0)	3/8 (38)	0.231	**0/9 (0)**	**4/6 (67)**	**0.011**
Enterobacteriaceae	3/10 (30)	3/3 (100)	0.070	**1/11 (9)**	**4/4 (100)**	**0.004**
Enterococcus	1/12 (8)	0/1 (0)	1.000	1/15 (7)	0/0 (0)	-
Finegoldia	1/3 (33)	8/10 (80)	0.203	0/3 (0)	8/12 (67)	0.077
Fusobacterium	0/10 (0)	1/3 (33)	0.231	**0/7 (0)**	**6/8 (75)**	**0.007**
Haemophilus	1/12 (8)	1/1 (100)	0.154	**1/13 (8)**	**2/2 (100)**	**0.029**
Lactobacillus	0/13 (0)	0/0 (0)	-	0/15 (0)	0/0 (0)	-
Parvimonas	0/11 (0)	1/2 (50)	0.154	1/10 (10)	2/5 (40)	0.242
Peptoniphilus	1/3 (33)	5/10 (50)	1.000	3/4 (75)	7/11 (64)	1.000
Porphyromonas	**0/8 (0)**	**3/5 (60)**	**0.035**	**0/11 (0)**	**4/4 (100)**	**0.001**
Prevotella	1/5 (20)	6/8 (75)	0.103	**1/4 (25)**	**10/11 (91)**	**0.033**
Sneathia	2/11 (18)	1/2 (50)	0.423	1/14 (7)	1/1 (100)	0.133
Staphylococcus	2/11 (18)	0/2 (0)	1.000	1/12 (8)	2/3 (67)	0.081
Streptococcus	6/13 (46)	0/0 (0)	-	6/13 (46)	2/2 (100)	0.267
Tissierellaceae 1-68	**1/11 (9)**	**2/2 (100)**	**0.038**	**0/11 (0)**	**4/4 (100)**	**0.001**
Tissierellaceae WAL_1855D	1/9 (11)	2/4 (50)	0.203	0/11 (0)	2/4 (50)	0.057
Veillonella	3/12 (25)	1/1 (100)	0.308	3/15 (20)	0/0 (0)	-

CHAPTER 9

General discussion and future perspectives

The human microbiota plays a critical role in health and disease (**Chapter 1**). 16S ribosomal RNA (rRNA) gene profiling provides the foundation for modern microbiota studies, boosting microbiota research and leading to a tremendous amount of publications exploring the possible role of the human microbiota in health and disease. This method can also be very valuable for the clinical microbiology because theoretically it enables detection and identification of an unlimited number of bacteria present in a specimen and permits (semi) quantitative information about the composition of a microbial community. Although 16S rRNA gene profiling is a very straightforward method its usability may be reduced due to its limited resolution to the genus level.

The aim of this thesis was to evaluate the clinical utility of 16S rRNA gene profiling, more specifically 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 existing diagnostic methods and therapies. In addition, we explored whether clinically relevant cut-off values for interpretation of the sequencing data could be defined, and what the limits are of using 16S rRNA gene profiling as a diagnostic tool.

To address this, studies focussing on different diseases were performed, including respiratory tract infections, bacterial vaginosis (a polymicrobial syndrome of the female urogenital system), atopic dermatitis (a chronic, inflammatory skin disorder associated with colonisation of the skin by *Staphylococcus aureus*) and male genital lichen sclerosus (a chronic lichenoid inflammatory fibrosing disorder of the male urogenital system with an unknown aetiology). Within these studies we applied 16S rRNA gene profiling as the main diagnostic tool or in combination with other commonly applied diagnostic methods. The main findings of the research performed are summarised and discussed in this final chapter. In addition, recommendations for future research are made.

16S rRNA GENE PROFILING AS A DIRECT DIAGNOSTIC TOOL

Lower respiratory tract infections (LRTIs) can be caused by a wide range of microorganisms, including bacteria that colonise the respiratory tract in health. For identification of bacterial pathogens involved in LRTIs, the standard algorithm involves culture of bacteria from sputum followed by species identification with matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) technology (1) and antibiotic-susceptibility testing of the cultured putative causative microorganism (2). The clinical diagnostic bacteriology is still dominated by culture-based methods despite the fact that culturing as stand-alone test provides limited insight into the polymicrobial community potentially present in a clinical specimen. While the MALDI-TOF MS technology has revolutionized clinical diagnostic bacteriology, this approach is dependent on culture and identification of potential pathogens may be hindered due to competition during selective culture and

the existence of non- or poorly-culturable pathogens, such as *Mycoplasma pneumonia*, *Chlamydia pneumoniae* and *Legionella pneumophila* (3). In **Chapter 2** we questioned whether a stepwise approach using 16S rRNA gene profiling followed by species-specific real-time quantitative PCRs (qPCRs) and/or culture has the potential to be a more accurate and efficient diagnostic approach than the routine diagnostic approach based on culture. We concluded that 16S rRNA gene profiling can be used to identify potential pathogenic genera in sputum, but only when combined with species-specific qPCR to achieve the needed resolution. This conclusion was based on the following observations, which will be discussed in more detail below:

i. 16S rRNA gene profiling provides a more complete characterisation of all potential pathogens in sputum than the routine culture-based approach, but the clinical interpretation of relative abundance of the different potential pathogenic genera remains a challenge.
ii. Classification to genus level based on the 16S rRNA gene is not sufficient to identify the causative microorganism of respiratory tract infections, requiring a second test to achieve the required resolution to the species level.
iii. Non- or poorly-culturable bacteria can be detected with 16S rRNA gene profiling.

Clinical interpretation of relative abundance of the different potential pathogenic genera

From 62 sputum samples, we identified a total of 110 potentially pathogenic genera with 16S rRNA gene profiling while only 37 pathogens were identified with the routine culture-based approach. This difference occurred because 16S rRNA gene profiling can detect all bacteria present in a specimen while culture is often more specific for a certain class of pathogenic species. Non-pathogenic species are not investigated by routine culture. Since both non-pathogenic and pathogenic species can colonise the respiratory tract without being involved in an infection (4-6), it is very important to provide a clinical interpretation of 16S rRNA gene profiling data. The current diagnostic approach based on culture already discriminates between infection and colonisation by using cut-off values defined in measures of colony counts or concentration of colony forming units (CFU) in association with the clinical syndrome (2, 7). For example, *Streptococcus pneumoniae* and *Haemophilus influenzae* are identified in sputum as the causative microorganism when ≥ 10 colonies are observed on an inoculated agar plate (7). 16S rRNA gene profiling data is expressed as a relative abundance (%) for each identified bacterial genus. As we had 16S rRNA gene and culture data available for our sputum samples, we compared this data in a first attempt to define clinically relevant cut-off values for 16S rRNA gene profiling. A wide range of relative abundances was found for the potential pathogenic genera *Streptococcus*, *Haemophilus* and *Moraxella*. For the genera *Streptococcus* and *Haemophilus*, relative abundances ≥ 25%

were observed in sputum for which *S. pneumoniae* or *H. influenzae* was designated as the causative organism by culture. For the genus *Moraxella*, a wide range of relative abundances (2-86%) were observed in sputm with *M. catarrhalis* as the causative organism according to culture. These data suggest (i) that a cut-off value of 25% relative abundance for the genera *Streptococcus* and *Haemophilus* can be used to screen with 16S rRNA gene profiling for infections caused by *S. pneumoniae* and *H. influenzae*, and (ii) that a cut-off value on relative abundance for the genus *Moraxella* is of no additional value. The difference in clinical interpretation of 16S rRNA gene profiling data for the genera *Streptococcus*, *Haemophilus* and *Moraxella* is most likely caused by the niche difference. In healthy adults, relative high abundances of *Streptococcus* and *Haemophilus* spp. are observed in the lower respiratory tract and the oropharynx, while *Moraxella* spp. are mainly found in the nasopharynx (4, 8). *M. catarrhalis* might reach the lungs by microaspiration from the nasopharynx where it is rapidly cleared or occasionally results in an infection, which occurs especially in individuals with chronic obstructive pulmonary disease (COPD) (9). This suggests that *M. catarrhalis* is only detected in sputum when involved in an LRTI. However, this is contradicted by the fact that *M. catarrhalis* is frequently cultured from sputum obtained from COPD patients without clinical evidence of an LRTI or exacerbation (9). *M. catarrhalis* might be less pathogenic than assumed.

Although we show that amplicon sequencing of the 16S rRNA gene can be used to screen for potential pathogenic genera in sputum, it is not sufficient to identify the causative microorganism. For the genera *Streptococcus* and *Haemophilus*, a relative abundance > 25% could also result from multiple colonising *Streptococcus* or *Haemophilus* species since relative abundance is based on the combined number of 16S rRNA genes from different species. To determine whether a potential pathogenic species is present and involved in a LRTI, identification and quantification at the species level is required e.g. by qPCR. Furthermore, although we were able to provide (not yet validated) clinically relevant cut-off values for some bacterial genera, an overall clinical interpretation of all the identified potential pathogenic genera within a polymicrobial community remains a challenge. This might delay a possible implementation of 16S rRNA gene profiling in clinical microbiology, which has been seen for whole genome shotgun (WGS) sequencing. WGS sequencing is the analysis of the complete DNA sequence of a single microorganism, enabling genotypic characterisation and investigation of genetic relationships between isolates (10-14). Besides, antimicrobial resistance genes can be detected. This is very valuable for clinical microbiology because it allows the hospital to identify the beginning of an outbreak of multi-drug resistant bacteria and take preventive measures to prevent the bacteria from further spreading. Despite this major advantage for clinical microbiology, implementation was delayed due to the complex data analysis and the challenge to define genetic distance cut-off values to detect outbreaks of different pathogens since bacteria evolve at different rates and replicate variably in different environments (15, 16).

Limited resolution of 16S rRNA gene profiling

It is important to emphasize that the commonly applied 16S rRNA gene profiling method targets a very small piece of conserved DNA (several hundred base pairs) to characterize polymicrobial communities. For most species within a single genus, the differences between species on this small piece of conserved DNA is limited to only a few or a single nucleotide and therefore remain indistinguishable. As a result, 16S rRNA gene data is in most cases reliable down to genus level and occasionally may provide species resolution. Classification down to genus level is not sufficient to identify the causative microorganism. Despite the fact that 16S rRNA gene profiling provides a more complete characterisation of polymicrobial communities, this limited resolution hampers its wide usage in clinical settings. There are several ways to push the classification of 16S rRNA gene profiling data to its limit, but it starts with selection of the 16S rRNA gene region with the highest amount of variation to enable discrimination between closely related bacteria. In general, the V1-V2, V3-V4 or V4 region is amplified and subsequently sequenced. To enable identification of the generated reads at species level, a database with high-quality 16S rRNA gene reference sequences with annotation down to species level is required. However, genus level is the lowest taxonomic group of the preferred SILVA database, which has the richest taxonomy of the available databases and is continuously updated (17). Assuming that the most optimal 16S rRNA gene region is targeted and that a high quality reference database is available, one way to achieve a substantial improvement in classification accuracy is by using a set of reference sequences that is specific for the sample's source environment. Popular classification pipelines assume that all species in a reference database are equally likely to be observed. Classification accuracy degrades linearly with the degree to which that assumption is violated, and in practice it is frequently violated. By incorporating environment-specific taxonomic abundance information, a significant increase in the species-level classification accuracy can be obtained (18). Another simple option would be to increase the length of the targeted 16S DNA since this also increases the amount of potential variation that is available for discriminating closely related bacteria. For example, amplifying and analysing the full length 16S rRNA gene instead of a small variable region of this gene may significantly increase discriminatory power. Currently, the Illumina Miseq platform is commonly used for 16S rRNA gene profiling. This technology generates short reads of 600 nucleotides as a maximum, which is insufficient to cover the full length amplified 16S rRNA gene of approximately 1540 nucleotides. For generating reads that cover the whole amplified 16S rRNA gene, a more recent developed platform is needed, such as the Pacific Biosciences (PacBio) or the Oxford Nanopore platform. Sequencing of the whole amplified 16S rRNA gene may have the discriminatory power to classify bacteria to the species level in spite of the technology's higher error rate (19, 20). Although third generation long read sequencing facilitates classification down to species level, for some very closely related species the full-length 16S rRNA gene might still be too conserved. For example, multiple *Streptococcus*

species can be present in the human respiratory tract, including the pathogen *S. pneumoniae* and *Streptococcus pseudopneumoniae* whose clinical importance is unknown. These species are phenotypically and genetically distinct from each other but their complete 16S rRNA genes differ only by a single nucleotide (21, 22). Accordingly, a second step is always required for identification of *S. pneumoniae* as the causative microorganism.

In our opinion, this second step should include a qPCR, which is a fast method that allows sensitive and specific detection as well as quantification of specific species. For *S. pneumoniae*, a concentration of 1.00E+05 gene copies/mL has been described as a significant cut-off value to identify *S. pneumoniae* as the causative microorganism (23). We found that screening with 16S rRNA gene profiling, using a relative abundance ≥ 25% as cut-off value, followed by a qPCR enabled identification of *S. pneumoniae* as the causative microorganism (**Figure 1a**). Sputum with a relative abundance < 25% for the genus *Streptococcus* and/or with a *S. pneumoniae* concentration < 1.00E+05 gene copies/mL were culture-negative and therefore we considered these to be not clinically relevant.

Similarly, multiple *Haemophilus* species can be present in the respiratory tract, including the pathogen *H. influenzae* and *Haemophilus parainfluenzae* whose clinical importance is debateable. We found that 12 of the 13 (92%) sputum samples with a relative abundance ≥ 25% for the genus *Haemophilus* had also a relatively high concentration of *H. influenzae* with a species-specific qPCR (≥ 3.35E+08 gene copies/mL). All 10 sputum samples for which *H. influenzae* was designated as the causative pathogen by culture belong to this group. Sputum with a relative abundance < 25% for the genus *Haemophilus* were culture-negative for *H. influenzae* and therefore we considered that these were not clinically relevant. Based on these data, we concluded that additional species determination and quantification by qPCR had no added value. In other words, applying the cut-off value for 16S rRNA gene profiling was sufficient to identify *H. influenzae* as the causative microorganism in our study (**Figure 1b**). Larger clinical studies are needed to confirm that identification and quantification at species level is redundant for sputum with ≥ 25% relative abundance of *Haemophilus*.

In contrast to *Haemophilus* and *Streptococcus*, we were unable to define a cut-off value on relative abundance for the genus *Moraxella* to screen with 16S rRNA gene profiling for infections caused by *M. catarrhalis*. A wide range of relative abundances for the genus *Moraxella* were observed in the culture-positive sputum for *M. catharrhalis*. For these sputum samples, we also observed relatively high concentrations (≥ 1.76E+08 gene copies/mL) of the pathogen *M. catarrhalis* by qPCR. Comparably high concentrations by qPCR were observed in all sputum samples that were culture-negative but positive for the genus *Moraxella* with 16S rRNA gene profiling. This suggests that other *Moraxella* spp. that may colonise the nasopharynx such as *Moraxella nonliquefaciens* were not present in the sputum specimens. Accordingly, a cut-off value on relative abundance for the genus *Moraxella* as well as species determination and quantification by qPCR is of no additional value for the identification of *M. catarrhalis* as the causative microorganism in this study (**Figure 1c**).

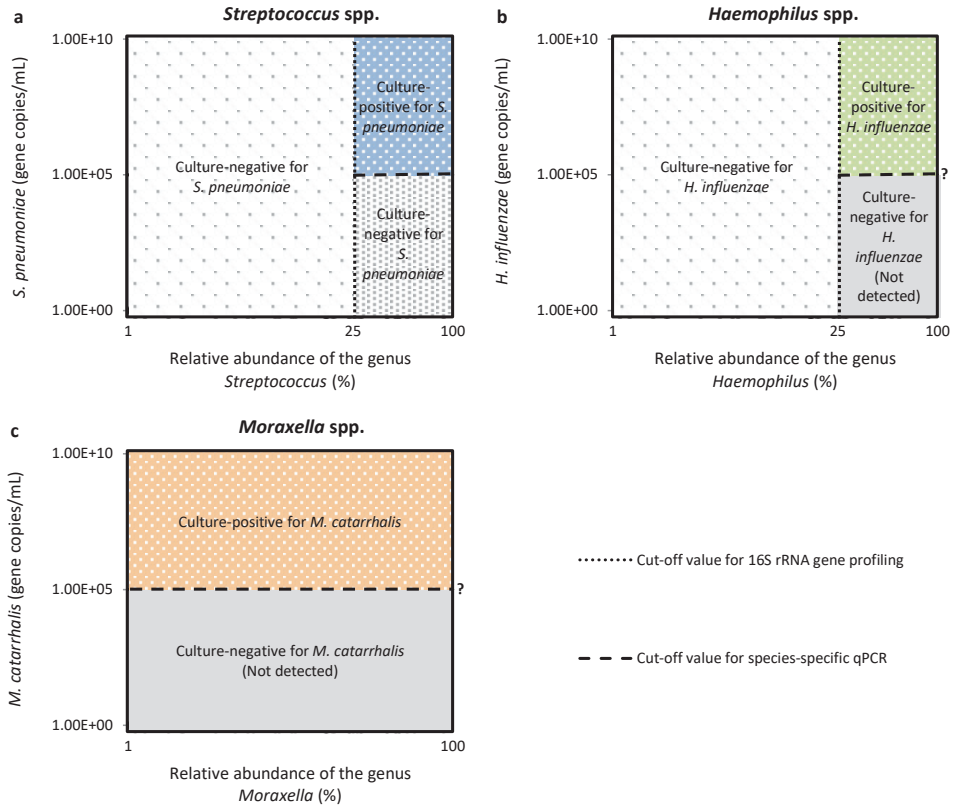


Figure 1. Clinical data interpretation of 16S rRNA gene profiling at the genus level (x-axis) and species-specific quantitative real-time PCRs (qPCRs; y-axis) of sputum is challenging as illustrated for (**a**) *Streptococcus* (**b**) *Haemophilus* and (**c**) *Moraxella* spp. For identification of *Streptococcus pneumoniae* as the causative microorganism (culture-positive; *blue*), a cut-off value for 16S rRNA gene profiling and for species-specific qPCR is required. For identification of *Haemophilus influenzae* as the causative microorganism (culture-positive; *green*), only a cut-off value for 16S rRNA gene profiling seems to be required. Sputum samples with a relative abundance above the cut-of value of 25% and a relatively low concentration of *H. influenzae* or negative by qPCR were not detected in this study (Culture negative; *grey*). For identification of *Moraxella catarrhalis* as the causative microorganism (culture-positive; *orange)*, detection of the genus *Moraxella* by 16S rRNA gene profiling seems to be sufficient. All sputum samples positive with 16S rRNA gene profiling for the genus *Moraxella*, irrespective of the relative abundance, were positive with the species-specific qPCR for *M. catarrhalis*. These sputum samples also included the culture-positive samples.

Although we highlighted that it is possible to optimize the classification of 16S rRNA gene profiling data to its limit, classification to the species level will not always be possible. For those genera, a species-specific qPCR can be used to acquire more precise diagnostic results. In contrast to culture-based methods, this stepwise approach is objective. Culture-based methods depend on the experience and subjectivity of the technician, because pathogens can have similar morphologic characteristics as non-pathogenic colonizers making their recognition by technicians difficult (24-26). Furthermore, the usefullness of culture-based methods is hampered by the existence of non- or poorly-culturable pathogens (3).

Non- or poorly-culturable bacteria

A major limitation of culture-based methods is the inability to culture all existing bacterial pathogens, such as the so-called 'atypical bacteria' *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila* (3). We detected the genus *Mycoplasma* in two sputum specimens with 16S rRNA gene profiling. One of these sputum specimens was positive for *M. pneumoniae* by qPCR, showing that 16S rRNA gene profiling is able to detect these bacteria. The other sample was negative for *M. pneumoniae* by qPCR, confirming that species-determination is required as a second step to discriminate between pathogenic and non-pathogenic species.

In clinical microbiology, microorganisms that are very difficult to culture are detected with PCR-based assays (2). To limit the number of tests to be performed, several PCR assays targeting different pathogens might be combined into one assay, called a multiplex PCR. Several multiplex panels are commercially available for respiratory tract infections, targeting not only (atypical) bacterial pathogens but also respiratory viruses, such as the Luminex xTAG respiratory viral panels (Luminex Molecular Diagnostics, Toronto, Canada), Respiratory Panel Assays of Seegene (Seoul, South Korea) and RespiFinder 2Smart (PathoFinder B.V., Maastricht, The Netherlands) (27-29). These panels are highly sensitive and specific for detection of a set of pathogens involved in LRTI. Multiplex species-specific qPCRs might be useful to target a large set of pathogenic species, but it is practically impossible to obtain a complete overview of a microbial community in a clinical specimen with this method. This highlights the additional value of 16S rRNA gene profiling for the routine clinical microbiology despite its lack of resolution.

An alternative method for the identification of pathogens in sputum is whole metagenome shotgun (WMS) sequencing. This technique offers an opportunity to identify and characterize bacterial species and other microorganisms, such as viruses, in a complex microbial community in relation to the microbiota (30). This method was recently used to find the causative agent of a severe new acute respiratory syndrome, which started in December 2019 in Wuhan, Hubei province of China (31). Health authorities identified a cluster of pneumonia cases linked to the city's South China Seafood Market at which a large

range of live or freshly slaughtered animals are sold including poultry and bats. Preliminary aetiological investigations excluded the presence of common respiratory pathogens using commercial assays, such as qPCR. Accordingly, metagenomic RNA sequencing (WMS sequencing on RNA level) was performed. This resulted in the identification of a new RNA strain of virus that was most closely related (89.1% nucleotide similarity) to a group of bat viruses of the family Coronaviridae. The new coronavirus was also phylogenetically close to severe acute respiratory syndrome coronavirus (SARS-CoV) and was therefore named 'SARS-CoV-2' (32). The World Health Organization (WHO) announced the official name of the disease as 'coronavirus disease 2019 (COVID-19)' Within a few months of the first report, SARS-Cov-2 had spread across China and worldwide, reaching a pandemic level. Measures taken to reduce its spread critically depend on timely and accurate identification of virus-infected individuals. Therefore, primers and probes were designed for qPCR assays based on the first sequence of SARS-CoV-2 generated with metagenomic RNA sequencing (33, 34). We implemented diagnostic assays in our labs as well as viral load and high throughput sequencing assays to support pharmaceutical companies trying to detect and treat COVID-19 (35-38).

We showed that 16S rRNA gene profiling can be used to identify potential pathogenic genera in sputum, but only when combined with species-specific qPCR to achieve the needed resolution. We do not believe that 16S rRNA gene profiling combined with species-specific qPCRs will completely replace culture. The major advantage of culture is the ability to characterise phenotypic aspects such as antibiotic susceptibility of a pathogen. Although there are exceptions such as *Mycobacterium tuberculosis*, phenotypic aspects of a pathogen cannot be predicted based on taxonomic classification or genotyping (39, 40). Accordingly, we would add selective culture to the combination of methods to be performed as a third step if indicated.

However, clinical microbiology will still prefer the routine culture-based approach and multiplex PCR-based methods above the combination of 16S rRNA gene profiling, qPCR and culture as it is cheap and relatively fast. The commonly used Illumina Miseq platform has a run time between 24 and 55 hours for sequencing amplicons ranging between 300 and 600 bp (41, 42). The relatively new Oxford Nanopore platform generates longer reads and has a relatively short run time of just a few hours, but more research is needed to confirm that higher error rates does not complicate 16S rRNA gene data analysis (20). Of note is that, in contrast to culture, faster, better and cheaper technology for sequencing can be expected in the near future (43).

When challenges of clinical interpretation of the data, sequencing turnaround time and costs are overcome, the combination of 16S rRNA gene profiling, qPCR and culture can be of interest for the clinical microbiology for the diagnosis of LRTI and urinary tract infections, though infections of body sites normally depleted of bacteria or with low diverse

microbiota are preferred, such as meningitis, septicaemia and prosthetic joint infections (44, 45). Nevertheless, comparable stepwise approaches will increase the diagnostic yield for detection of pathogenic species involved in those type of infections (44, 45).

16S rRNA GENE PROFILING AS ALTERNATIVE REFERENCE TEST FOR EVALUATION OF DIAGNOSTIC ALGORITHMS

Infections such as bacterial vaginosis (BV) are not caused by a single microorganism but involve (complete) disturbance of the microbial community. BV is characterised by a shift from a *Lactobacillus* spp. dominated vaginal microbiota to a more diverse microbiota causing a malodorous vaginal discharge (46). The 2018 European International Union against Sexually Transmitted Infections (IUSTI) World Health Organisation (WHO) guideline on the management of vaginal discharge recommends diagnosing BV using clinical symptoms and signs and bedside tests, supported by laboratory test findings (47). However, no diagnostic algorithm is proposed but instead all options are presented. Amsel's clinical criteria (48), Nugent score (49), culture-based techniques (46) or CE-IVD marked qPCRs (50, 51) are commonly used methods for the diagnosis of BV. These methods have been extensively evaluated using the Nugent score as the gold standard (50-55). The Nugent score is a Gram stain scoring system for vaginal swabs based on the quantitative assessment of *Lactobacillus* spp. morphotypes (decrease scored as 0 to 4), *Gardnerella vaginalis* morphotypes (increase scored as 0 to 4) and *Mobiluncus* spp. morphotypes (increase scored as 0 to 2) (49). A score of 7 to 10 is consistent with BV. It is known that some small bacterial morphotypes may vary in size and form, and sometimes are difficult to distinguish them from *Lactobacillus* spp. morphotypes (56, 57). This phenomenon makes the interpretation of the Nugent score subjective. Adoption of the Nugent score by clinical laboratories is limited by its complexity and subjectivity (58). Furthermore, the proportion of samples assed with an intermediate score between 4 and 6 may exceed 20% and it remains debated how to treat these patients (56, 59-61). Hence, we emphasize the need for an objective reference method. In **Chapter 3**, we explored using 16S rRNA gene profiling as an alternative reference test to evaluate existing tests for the diagnosis of BV. To discriminate between a normal vaginal microbiota and BV, we used a cut-off value of 47% relative abundance of the genus *Lactobacillus*, which has previously been reported as an accurate BV predictor (62, 63). *Lactobacillus* spp. dominated vaginal microbiota profiles (with ≥47% relative abundance of the genus *Lactobacillus*) were categorised as normal vaginal microbiota and microbiota profiles with less *Lactobacillus* (<47% relative abundance) with mainly anaerobes as microbiota associated with BV. Based on the following findings, we consider 16S rRNA gene profiling as a good alternative to replace Nugent score, the current gold standard, to evaluate BV diagnostic methods:

i. Poor sensitivity of the Nugent score to diagnose BV with 16S rRNA gene profiling as reference test.
ii. A cut-off value of 47% relative abundance for the genus *Lactobacillus* is highly accurate for identification of women with a normal vaginal microbiota.
iii. The definition <47% relative abundance of the genus *Lactobacillus* and a higher relative abundance of anaerobic bacteria compared to aerobic bacteria is sufficient to identify women with BV, but more clinical studies are necessary.

Performance of the Nugent score (current gold standard) for the diagnosis of BV

Despite its complexity, the Nugent score has almost exclusively (or combined with Amsel's clinical criteria) been used to evaluate other methods to diagnose BV (50-55). Furthermore, preliminary diagnosis may be based on Amsel's clinical criteria, but the Nugent score remains the definitive laboratory method for diagnosis (2). A simplified version of the Nugent score has been described, called the Hay/Ison criteria (64). Instead of the complex scoring system, vaginal swabs with predominantly *Lactobacillus* morphotypes are assigned to grade 1 (normal vaginal microbiota), mixed flora with some *Lactobacillus* and *Gardnerella* or *Mobiluncus* morphotypes to grade 2 (intermediate flora), and *Gardnerella* and/or *Mobiluncus* dominated morphotypes to grade 3 (BV). These simpler criteria have been found to correlate well with Amsel's clinical criteria as well as with the Nugent score (65, 66). It may be an alternative for the Nugent score (2). However, assigning vaginal swabs to the different grades remains subjective and especially interpretation of the intermediate score remains questionable (67).

Since the development of the Nugent score in 1991, more advanced technology has become available. To the best of our knowledge, we were the first to determine the current performance of the Nugent score. We found a specificity of 100% and a sensitivity of 64% for the diagnosis of BV using 16S rRNA gene profiling as the reference test. This means that the Nugent score is highly accurate for identification of women without BV but that it misses 36% of the women with BV. Evaluation of the discrepant results between 16S rRNA gene profiling and the Nugent score showed that interpreting the intermediate score as BV negative was mainly responsible for the poor sensitivity. Interpreting the intermediate Nugent score as BV positive would, however, result in an increased sensitivity of 92% and a substantial decreased specificity of 87%. Half of the number of swabs with an intermediate Nugent score were categorised as microbiota associated with BV (relative abundance between 0% and 36% for the genus *Lactobacillus* and between 100% and 64% for anaerobes) and the other half were categorised as normal vaginal microbiota (relative abundance between 50% and 99% for the genus *Lactobacillus* and between 50% and 1% for anaerobes). Due to the wide range of *Lactobacillus* abundance observed in the intermediate category, the clinical importance of the intermediate category remains unresolved (56, 59-61, 67). Therefore, the Nugent score as the gold standard should be replaced by a method with a clear definition

of vaginal health and BV. We propose 16S rRNA gene profiling as an alternative reference test for the diagnosis of BV.

Definition of normal vaginal microbiota

To be able to use 16S rRNA gene profiling as a reference test for the diagnosis of BV, a reliable cut-off value for the relative abundance of the genus *Lactobacillus* is required to discriminate between normal vaginal microbiota and microbiota associated with BV. A general definition of normal vaginal microbiota is a predominance of *Lactobacillus* spp. (68-72). It remains to be discussed what level of *Lactobacillus* abundance defines a healthy or a BV associated vaginal microbiota (**Figure 2**). We found one report that investigated the composition of the vaginal microbiota in healthy women and women with BV (62). In this paper, a 16S rRNA gene profiling cut-off value is proposed for the diagnosis of BV. To evaluate their cut-off value of 47% relative abundance of *Lactobacillus*, we compared the outcome of five diagnostic methods (i.e. Amsel's clinical criteria, Nugent score, culture and 2 CE-IVD marked qPCRs) with 16S rRNA gene profiling. All five diagnostic methods were in agreement with 16S rRNA gene profiling for at least 92% of the swabs categorised as normal vaginal microbiota. Analysis of the discrepant test results showed that for the discrepancies found, not all five diagnostic methods agreed on the test result. This means that these particular samples were very complex to interpret and not necessarily a discrepancy solely by the reference test in question. As such we conclude that a cut-off value of 47% relative abundance of the genus *Lactobacillus* is highly accurate to identify women with normal vaginal microbiota (≥ 47%).

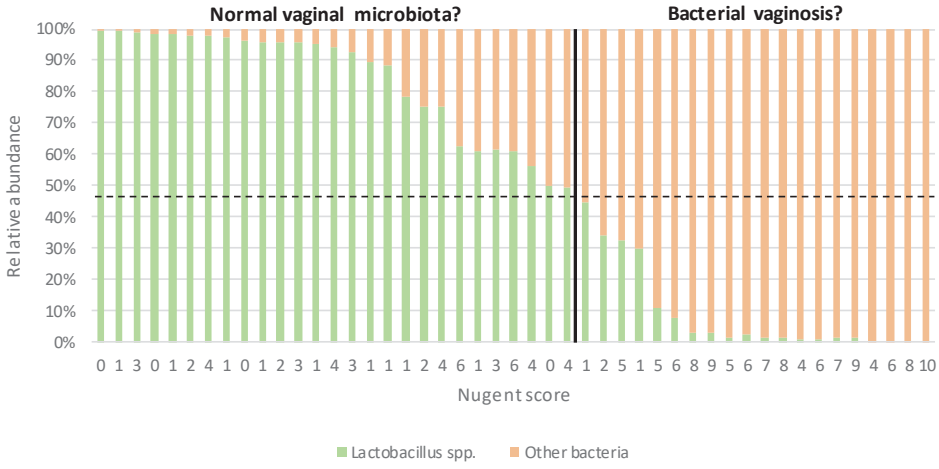


Figure 2. Defining a cut-off value (dotted line) for the genus *Lactobacillus* in measures of relative abundances (y-axis) to discriminate between normal vaginal microbiota and bacterial vaginosis is challenging as illustrated by the Nugent score (x-axis). A Nugent score of 0 to 3 is consistent with normal flora, 4 to 6 with intermediate flora and 7 to 10 with bacterial vaginosis.

We noticed that 16S rRNA gene profiling as well as the five applied diagnostic methods do not discriminate between various *Lactobacillus* spp. needed for the identification of women with normal vaginal microbiota or BV. The healthy vagina is most frequently dominated by one, or at the most two species of Lactobacilli from a short list of four: *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii* or *Lactobacillus iners* (72). In contrast to other *Lactobacillus* spp., *L. iners* is commonly found in the vagina of women with BV (73-76). This may be because *L. iners* is better adapted to the changing conditions associated with BV, such as the polymicrobial state of the vaginal microbiota and elevated pH (74). However, only 9% of *L. iners* strains produce hydrogen peroxide, which has antimicrobial properties. This is low compared to the 94%, 95% and 70% of the *L. crispatus*, *L. jensenii* and *L. gasseri* strains, respectively (77). Furthermore, *L. iners* strains do not produce high quantities of lactic acid and thus may fail to acidify the vagina to low pH to suppress the growth of pathogenic microorganisms (78, 79). Accordingly, it has been suggested that when the microbiota is dominated by *L. iners*, it is more likely to shift towards dysbiosis unlike when *L. crispatus* is dominant (80). At present, the available literature is insufficient to classify *L. iners* as a 'friend' or 'foe' (81). Further clarification of its role in health and disease is warranted in the future.

Definition of microbiota associated with BV

Although we show that a cut-off value of 47% relative abundance for the genus *Lactobacillus* is highly accurate to identify women with normal vaginal microbiota (**Figure 3a**), it is not sufficient as a stand-alone criterion to identify women with BV. This cut-off value allows differentiation between normal vaginal microbiota and dysbiosis but it does not distinguish between BV and another bacterial vaginal infection, called desquamative inflammatory vaginitis or aerobic vaginitis (AV) (82-84). Both conditions are associated with a wide spectrum of bacteria, but BV is characterised by an increase in anaerobes (68-72) whereas AV is defined by an increase in aerobes (82), such as *Escherichia* and *Streptococcus* spp. Accordingly, we defined microbiota associated with BV as <47% relative abundance of the genus *Lactobacillus* and mainly anaerobes, and AV as <47% relative abundance of the genus *Lactobacillus* and mainly aerobes. It should be noted that there is ongoing discussion whether AV is a separate entity from BV (61).

To evaluate the definition of BV for 16S rRNA gene profiling, we compared our 16S rRNA gene profiling data with the outcome of Amsel's clinical criteria, Nugent score, culture and 2 CE-IVD marked qPCRs (**Figure 3b**). At least two methods were in agreement with 16S rRNA gene profiling in 81% of the swabs categorised as microbiota associated with BV. Their microbiota profiles were depleted from the genus *Lactobacillus* and dominated by the strongly BV-associated bacterial genera *Gardnerella*, *Atopobium* and/or *Prevotella* (46, 57, 85-87). The microbiota of the remaining swabs with discrepant results were mainly characterised by a relatively high abundance of bacteria that are less strongly associated with BV and/or not commonly found in the vagina. Most noteworthy is the genus

Bifidobacterium because of its proposed association with vaginal health (56, 88, 89). When *Bifidobacterium* spp. are confirmed to be associated with vaginal health, the definition of BV shas to be adjusted with presence of either *Lactobacillus* and/or *Bifidobacterium* spp. Another suggested alternative is to combine the cut-off value for the genus *Lactobacillus* with the presence of a limited number of bacteria that are strongly associated with BV, such as *Gardnerella* and *Atopobium* spp. (62). The disadvantage of this approach is that swabs with microbiota profiles dominated by other BV-associated anaerobes, such as *Prevotella* spp., would be incorrectly categorised. This illustrates that diagnosis of BV is difficult due to the wide spectrum of anaerobes associated with BV and the limited number of bacteria that can be effectively cultured or targeted by qPCR.

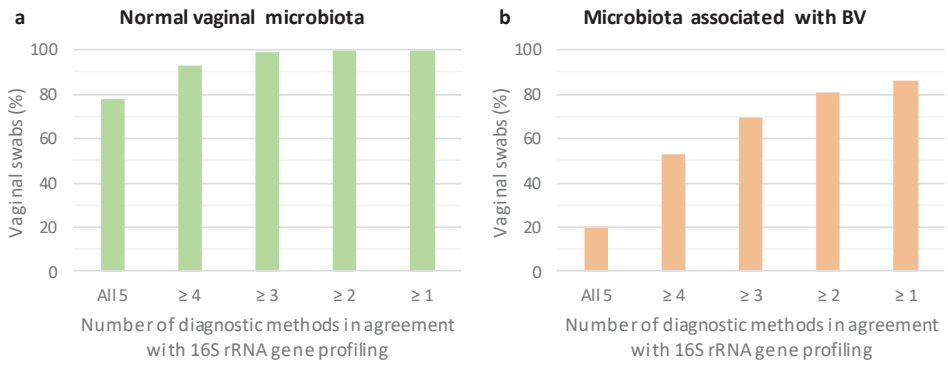


Figure 3. Defining (**a**) normal vaginal microbiota is less complicated than (**b**) microbiota associated with bacterial vaginosis (BV) as illustrated by the outcome comparison of 16S rRNA gene profiling and five diagnostic methods (Nugent score, Amsel's clinical criteria, culture and two CE-IVD quantitative real-time PCRs). Vaginal swabs with ≥47% relative abundance of the genus *Lactobacillus* were categorised as normal vaginal microbiota and vaginal swabs with <47% relative abundance and predominant anaerobes as microbiota associated with BV. For each category, the fraction of vaginal swabs (y-axis) for which all 5, ≥ 4, ≥ 3, ≥ 2 or ≥ 1 diagnostic method was in agreement with 16S rRNA gene profiling (x-axis) was plotted.

Based on these observations, we consider 16S rRNA gene profiling as a good alternative for the current golden standard to evaluate BV diagnostic methods. We found that the cut-off value of 47% relative abundance of the genus *Lactobacillus* is highly accurate to identify women with a normal vaginal microbiota. Currently, the definition <47% relative abundance of the genus *Lactobacillus* and predominance of anaerobes is sufficient to identify women with BV, but clinical studies with well-defined controls are required to identify the role of organisms such as *Bifidobacterium* spp. in the vaginal microbiota. This illustrates that it is challenging to provide a strict definition for microbiota associated with BV. This is due to: (i) a substantial number of asymptomatic women with disturbed vaginal microbiota (72) and (ii) the existence of other fungal and protozoal vaginal conditions associated with

complaints of abnormal vaginal discharge (47) such as vulvovaginal candidiasis (VVC) and vaginal infection with *Trichomonas vaginalis*. For other polymicrobial diseases it might be even more challenging to establish a cut-off value to discriminate between health and disease. Healthy microbiota of other body sites, such as skin, are even more variable between healthy individuals due to environmental factors, such as hygiene, lifestyle, geographic location, medication and diet (90-93).

HOW TO APPLY 16S rRNA GENE PROFILING TO DETERMINE THE IMPACT OF TREATMENT ON POLYMICROBIAL COMMUNITIES OF THE VAGINA AND THE SKIN

So far, we have discussed the application of 16S rRNA gene profiling as a direct diagnostic tool for the identification of clinically relevant microorganisms and as an alternative reference test for evaluation of existing diagnostic methods. Here, we will discuss the usefulness of 16S rRNA gene profiling as a tool to determine the impact of treatment on polymicrobial communities. Firstly, we focused on the treatment of BV with antibiotics because up to 40% of the patients treated for BV return to their physician with persistent complaints of abnormal vaginal discharge (94, 95). 16S rRNA gene profiling might be valuable for studying why treatment failure occurs in these patients by comparing the vaginal microbiota before and after treatment. Next, we shifted our focus to atopic dermatitis (AD) and studied if 16S rRNA gene profiling can be applied in clinical trials. AD, also known as atopic eczema, is a chronic, inflammatory skin disorder associated with colonisation of the skin by *Staphylococcus aureus* (96, 97). The cause of AD is unknown but believed to involve genetics, immune system dysfunction, environmental exposures, and changes of the structure of the skin. The disease may occur at any age, but typically starts in childhood and is chronic with swings in severity. New drugs to treat AD are being developed because of the limitations of emollients (non-cosmetic moisturisers/barrier creams) and topical anti-inflammatory corticosteroids (98-100). For clinical trials assessing the effectivity of the new drugs in patients with AD, the skin microbiota is an interesting biomarker (101). The relative abundance of *S. aureus* seems to be correlated with the severity of AD (96, 97), suggesting that restoration of the normal diverse skin microbiota is effective for treating AD. We concluded that 16S rRNA gene profiling has potential for studying the impact of treatment on polymicrobial communities. This conclusion was based on the following observations:

I. 16S rRNA gene profiling contributes to better understanding as to why women return to their physician with persistent complaints of abnormal vaginal discharge (**Chapter 4 and 5**).
II. Microbiota stratification of the skin with 16S rRNA gene profiling is useful to analyse treatment effects in AD trials (**Chapter 6**).

16S rRNA gene profiling contributes to better understanding of treatment failure in women with BV

As discussed previously, diagnosis of BV is challenging due to the subjective nature of Nugent scoring, the wide spectrum of anaerobes associated with BV and the limited number of bacteria that can be effectively cultivated or targeted by qPCR. This might result in misdiagnosis and subsequent women returning to their physician with persistent complaints of abnormal vaginal discharge (94, 95, 102). Comprehensive analysis of the microbiota before and after treatment is only useful when the persistent complaints are caused by incomplete restoration of the vaginal microbiota and not by misdiagnosis. In **Chapter 4**, women with complaints of abnormal vaginosis were diagnosed and subsequently treated according to the standard protocol of the hospital. A fraction of these women returned to their physician with persistent complaints of abnormal vaginal discharge. We determined whether misdiagnosis was the main reason for these persistent complaints. Since complaints of abnormal vaginal discharge may be caused by other microorganisms, 16S rRNA gene profiling as well as fungal culture and a qPCR were performed to diagnose bacterial infections such as BV, AV, VVC and *Trichomonas vaginalis* infection. Comparison of the data obtained during two subsequent visits showed that misdiagnosis was the cause for 30% women who returned with persistent symptoms. In another 30% of the women, the emergence of a different infection or failure of the treatment to restore the vaginal microbiota was the cause of the persistent symptoms (103-106). These data show that 16S rRNA gene profiling contributes to better understanding why women return to their physician with persistent complaints of abnormal vaginal discharge, but that it is not sufficient as a stand-alone test.

In **Chapter 5**, we performed comprehensive analysis on the 16S rRNA gene profiling data obtained from the patients with clinically diagnosed BV to elucidate why treatment fails to restore the vaginal microbiota in some women. We observed two bacterial community types before and after antibiotic treatment with significantly different bacterial diversity. Of the community types identified in women before treatment, one was driven by the genera *Lactobacillus*, *Gardnerella* and *Atopobium*, and the other one was driven by the genera *Gardnerella*, *Atopobium*, *Prevotella* and *Sneathia*. Antibiotic treatment allowed *Lactobacillus* to recover at the expense of *Atopobium*, but *Lactobacillus* did not become the dominant genus in the vaginal microbiota of all treated women. The two community types identified after treatment were either driven by *Lactobacillus* or by multiple bacteria. Unfortunately, we found no association between the community types before or after treatment and the clinical outcome (defined as no or persistent symptoms). Instead, we hypothesize that the presence of specific bacterial strains, human genetics and/or a microbiota function (which bacterial genes are present) may be associated with treatment failure. Recent advances in BV pathogenesis research have suggested distinct roles for the *G. vaginalis* clades (107-110). It may be that *G. vaginalis* strains with low virulence potential are able to colonise healthy women but are not involved in BV development, which might explain the high prevalence

of this genus in asymptomatic women. Only *G. vaginalis* strains able to overgrow healthy vaginal *Lactobacillus* spp. and initiate biofilm formation on the vaginal epithelium may be virulent. Biofilm formation has been proposed to be a crucial step in BV development as it represents a protective mode of growth that allows other anaerobes to survive in the acidic vaginal environment (85). Furthermore, biofilm formation by *G. vaginalis* contributes significantly to BV treatment failure and high recurrence rates (111-113). Since only specific strains of *G. vaginalis* may be involved in BV development, it might be that the presence or combinations of specific bacterial strains in the biofilm are responsible for treatment failure and recurrence (114-120). The 16S rRNA gene lacks the resolution to differentiate between strains with high and low virulence potential, which is a major disadvantage for 16S rRNA gene profiling.

Taken together, 16S rRNA gene profiling contributes to a better understanding of why women return to their physician with persistent complaints of abnormal vaginal discharge but lack resolution to discriminate between virulent and non-virulent bacterial strains. An alternative approach would have been to use WMS sequencing to study treatment failure. As previously discussed, WMS sequencing enables identification of the different bacteria as well as other microorganisms, such as fungi and protozoa, to the species or strain level. In this case, classification down to strain level is required, which cannot always be achieved with WMS sequencing. WMS sequencing also results in relative abundance information for all genes present in a vaginal swab, which can be used to generate the virulence profile of the polymicrobial community present in a swab (30). The difference in virulence profile before treatment between responders and non-responders might elucidate the mechanism behind treatment failure. Unfortunately, this method is not yet ready for clinical microbiology.

Microbiological phenotype stratification with 16S rRNA gene profiling and frequent sampling is required to analyse the microbiota in clinical atopic dermatitis trials

In **Chapter 6**, we further explored the utility of 16S rRNA gene profiling to determine the impact of treatment on the microbiota. This time, we focussed on how 16S rRNA gene profiling can be applied in clinical trials assessing the effectivity of new drugs in patients with AD. For these clinical trials, the skin microbiota is an interesting biomarker since AD is associated with *S. aureus* colonisation and reduced microbial diversity (101). Accordingly, new treatments are increasingly evaluated using clinical AD scores and the skin microbiota composition (101, 121-124). The microbiota composition of healthy skin may vary significantly between humans (inter-individual variation) and within a human over time (intra-individual variability) due to host and environmental factors, such as antibiotic exposure, hygiene and lifestyle (92, 93, 125-127). Importantly, skin affected by AD is likely to have larger inter- and intra-patient variability of the skin microbiota, implying the need for frequent sampling when evaluating the impact of treatment on the affected skin microbiota. However, to our knowledge, most clinical trial designs have included a

single sample before and after treatment. We questioned whether this limited sampling is sufficient to capture the full extent of skin microbiota variability. Baseline characterisation of the skin microbiota with 16S rRNA gene profiling confirmed the reduced microbiota diversity and increased relative abundance of the genus *Staphylococcus* for affected skin compared to unaffected skin. Furthermore, we observed a correlation between the relative abundance of the genus *Staphylococcus* and the concentration of *S. aureus* determined by culture and qPCR. This confirmed that, as expected, the relative abundance of *S. aureus* was higher on affected skin compared to unaffected skin. To measure the inter- and intra-patient variability over a period of 42 days, the standard deviation of the mean was calculated for microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration. For affected skin, we observed a high inter- and a wide range of intra-patient variability for all test results in the range of 36-94% and 7-173%, respectively. In a second independent patient group, we observed comparable inter- and intra-patient variability for affected skin. Since the sampling method was strictly standardized, the observed variability is unlikely to be caused by variable sample quality but rather reflect a highly variable and personalized profile. Because the variability over time can be high, limited sampling is not sufficient to determine the impact of the treatment on an individual's affected skin microbiota (**Figure 4**). Frequent sampling during intervention and statistical analyses methods which use repeated measures across more than one end of study time point, may reduce the effect of the variability in the analyses of clinical trials.

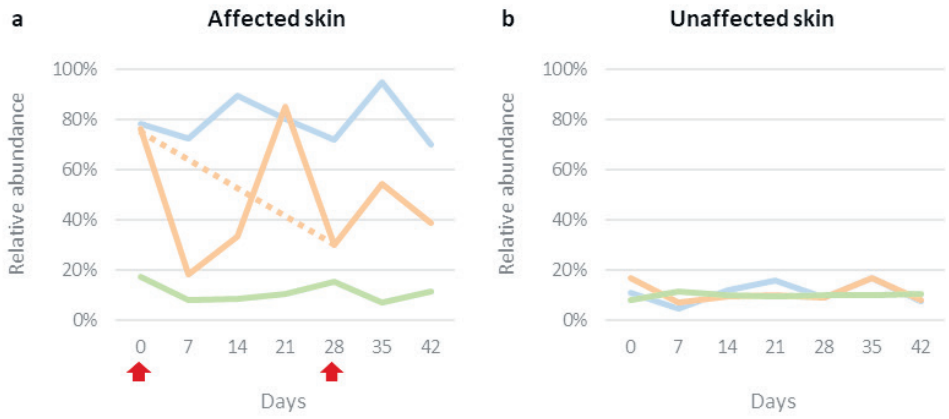


Figure 4. Limited sampling is insufficient to capture the full extent of skin microbiota variability in patients with atopic dermatitis as illustrated by the relative abundance of the genus *Staphylococcus* (x-axis)over time (y-axis) for (**a**) affected and (**b**) unaffected skin of patients with microbiological phenotype I (*blue*), II (*orange*) or III (*green*). In a clinical trial, the relative abundance of *Staphylococcus aureus* is an interesting biomarker and would be compared at day 0 and 28 (red arrows) to determine the impact of treatment. For the patient with phenotype II, this comparison would show that the relative abundance of *Staphylococcus* decreased (dotted line) while there was also an increase in the relative abundance of *Staphylococcus* between the two timepoints.

In addition, we were able to define three patient groups with different microbiological phenotypes. We categorized these patients according to their microbial diversity of skin microbiota, the relative abundance of the genus *Staphylococcus*/*S. aureus* and microbiota variability in time (**Table 1, Figure 4**). The impact of treatment on the skin microbiota might differ between the three phenotypes. Therefore, it is important to include the existence of the different phenotypes in the design of a clinical trial e.g. by including microbiological phenotype stratification with 16S rRNA gene profiling before intervention.

Table 1. Description of the three different microbiological phenotypes of lesional skin

Microbiological phenotype	Microbial diversity	Relative abundance of the genus *Staphylococcus*	Concentration *S. aureus*	Microbiota variability over time
I	Low	High	High	Low
II	Low	High	High	High
III	High	Low	Low	Low

In summary, we showed that 16S rRNA gene profiling contributes to a better understanding of why women return to their physician with persistent complaints of abnormal vaginal discharge and that it has a high potential to be applied in clinical trials to determine the impact of treatment on polymicrobial communities of the skin. Our progressive understanding of the human microbiota and its association with human disease has led to the considerable need for improved therapies, meaning that we foresee a huge increase in clinical trials for which the human microbiota can be an interesting biomarker. For the study design and accurate interpretation of the data, knowledge regarding microbiota variability and microbial phenotypes is mandatory.

Translation of research into the clinic

Previously, we explored whether 16S rRNA gene profiling has added value to clinical microbiology as a direct or indirect diagnostic tool. To address this, studies focussing on different diseases were performed, including BV and AD. Currently, human microbiota research linked to other diseases is increasing exponentially. However, we prefer more fundamental research before microbiota findings can be safely and widely applied into the clinical setting. To illustrate this, we used 16S rRNA gene profiling as a research tool to study the link between the microbiota and two different medical conditions.

In **Chapter 7**, we explored whether specific nasal and/or oropharyngeal microbiota profiles can be associated with increased age and RTIs in adults. This study was performed to provide insight as to why elderly (≥65 years) are more susceptible to RTIs (128, 129). We defined eight nasal and nine oropharyngeal microbiota clusters. Unfortunately, we were unable to clarify why elderly are more susceptible to RTIs. We showed that nasal microbiota

dominated by the genus *Moraxella* (presumably *Moraxella nonliquefaciens*) is associated with respiratory health in the elderly population. The nasal microbiota cluster dominated by the genus *Moraxella* was significantly more prevalent in the healthy elderly population ($p = 0.002$) compared to the healthy middle aged adults, and it was significantly less prevalent in elderly patients with a LRTI ($p = 0.001$) compared to the healthy elderly population. Interestingly, in young children (<5 years), which form another population at risk for RTIs, *Moraxella* spp. become predominant nasal community members over time (130-134). Later in life, other bacteria take their place as predominant community members. In young children, their microbiota as well as their immune system are still immature, whereas the immune system of the elderly deteriorates back towards an immature state (135). In essence, it might tolerate the same bacterial species. This might explain the significantly higher prevalence of *Moraxella* spp. in the healthy elderly population. However, conflicting results have been reported regarding the role of *Moraxella* spp. in the pathogenesis of RTIs in young children. Profiles dominated by *Moraxella* spp. such as *M. catarrhalis* or *M. nonliquefaciens* was associated with respiratory health (130-133). Others reported that *Moraxella* spp. such as *M. catarrhalis* were associated with high susceptibility to LRTIs (134). We found that the microbiota profiles within the relevant cluster were represented by *M. catarrhalis* and *M. nonliquefaciens*. Since *M. catarrhalis* has been considered as a pathogen for diseases such as COPD and otitis media (9, 136), and only represented 18% of the microbiota profiles dominated by the genus *Moraxella*, we hypothesize that *M. nonliquefaciens* is associated with respiratory health in both young children and elderly. However, some caution is necessary when translating this research based on 16S rRNA gene profiling into the clinical setting. For young children, the results were based on microbiota data obtained during longitudinal studies. For the elderly, we collected data at one timepoint, meaning that we cannot distinguish whether *M. nonliquefaciens* was less prevalent in elderly patients due to its protective nature or that the infection changed the microbiota. Accordingly, fundamental research is required to explore the protective properties of *M. nonliquefaciens*. When longitudinal studies and fundamental research confirm that *M. nonliquefaciens* is beneficial in relation to RTI, efforts should be made to uphold these beneficial bacteria by using narrow-spectrum instead of broad-spectrum antibiotics as much as possible (137). *M. nonliquefaciens* might even be a possible *Candida*te for probiotic therapeutic interventions against RTIs.

In **Chapter 8**, we explored whether the balanopreputial and urine microbiota are associated with a chronic inflammatory, scarring dermatosis associated with penile cancer, called male genital lichen sclerosus (MGLSc). It has been hypothesised that MGLSc arises from occluded exposure of a susceptible epithelium to urine since (i) MGLSc is exceptionally rare in males circumcised at birth, (ii) circumcision is usually curative and (iii) the association of MGLSc with high rates of post-micturition micro-incontinence (138-140). Circumcision dramatically changes the penile microbiota composition (141, 142),

suggesting that dysbiosis may play a role in the aetiology and pathogenesis of MGLSc. For the balanopreputial sac, we observed a difference in microbiota profiles between men with MGLSc and controls. The relative abundance of the genus *Finegoldia* was decreased in men with MGLSc (median relative abundance of 9% vs 28%) while the relative abundance of the genus *Prevotella* was increased (median relative abundance of 20% vs 4%). Both the prevalence (50% vs 15%) and relative abundance (median relative abundance of 4% vs 0%) of the genus *Fusobacterium* were increased in men with MGLSc. *Fusobacterium* spp., particularly *Fusobacterium nucleatum*, have been associated with inflammatory diseases such as periodonitis and inflammatory bowel disease and some cancers such as colorectal and oral squamous cell carcinoma (143-148). These observations suggest that dysbiosis of the balanopreputial sac microbiota is involved in MGLSc. However, we were unable to draw strong conclusions from this study due to the low number of included patients (n = 40) and the inability to classify to species level. In addition, longitudinal data is lacking to establish whether dysbiosis of the balanopreputial sac microbiota is the cause or consequence of the disease. Before anything can be translated into the clinical setting, larger powered case-control studies are required. Thereafter, involvement of balanopreputial sac dysbiosis in the aetiology and pathogenesis of MGLSc, with a specific role for *F. nucleatum*, should be confirmed by reproduction of the disease *In vitro* or in animal models. In contrast to *M. nonliquefaciens*, fundamental research to explore the protective or pathogenic properties of *F. nucleatum* have already been performed for its role in other diseases (149-157).

Both studies illustrate that translation of microbiota research into the clinical setting starts with finding a possible epidemiological link between the human microbiota and a specific disease with a pilot study (**Figure 5**). The possible link should be confirmed in large, well-powered epidemiologic studies with well-defined controls. Thereafter, multiple fundamental studies are required to explore the protective or pathogenic properties of a bacterial species, its interactions with other bacteria and the host, and to replicate the

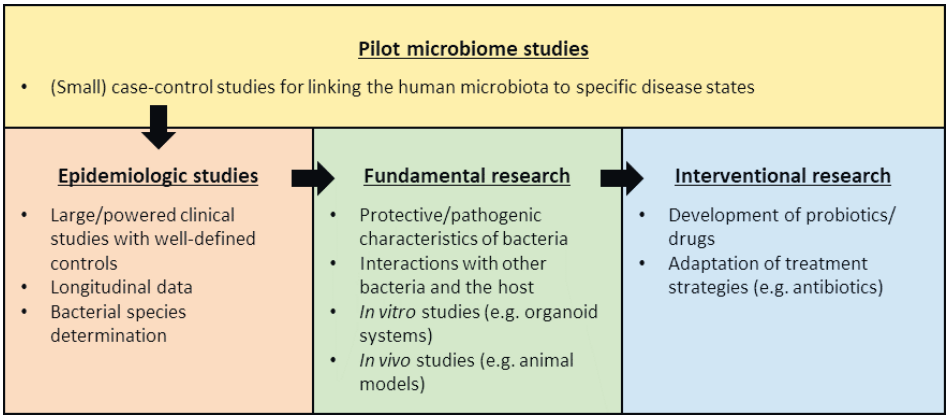


Figure 5. Translation science

disease in an *In vitro* or animal model. When the role of a bacterial species in a specific disease is revealed, interventional research can be performed to develop probiotics to prevent or drugs to treat specific diseases. This is the pathway that should be taken to translate microbiota research into the clinic.

FUTURE PERSPECTIVES

The main challenge of 16S rRNA gene profiling is the clinical interpretation of relative abundance of the identified bacteria in clinical specimens, such as sputum, urine and vaginal, skin, nasal and oropharyngeal swabs. More clinical studies with appropriate control groups are needed to define and validate clinically relevant cut-off values, to measure microbiota variability over time and to determine microbial phenotypes. However, each step of the 16S rRNA gene profiling method can influence the interpretation of the result. The transport and storage conditions after a specimen has been collected can have an impact on the DNA yield and DNA quality (158-161) and the choice of DNA extraction kit influences the results as some cell types may resist common mechanical or chemical lysis methods (162-165). Selection of primers to amplify the 16S rRNA gene is probably the most discussed issue (19). Universal PCR primer sets are designed to amplify as many different 16S rRNA gene sequences from a wide range of bacterial species as possible. However, there are no suitable 100% conserved regions of the 16S rRNA gene available for PCR amplification. This can lead to inaccurate microbiota profiles due to inefficient PCR primer binding (166). Another pitfall is that different bioinformatics pipelines, analysis settings and reference databases can affect the final microbiota results obtained (167, 168). Last but not at least, contaminant DNA derived from the environment, reagents and/or consumables used during sample processing can bias microbiota results. This is particularly relevant for studies with low microbial biomass specimens, since even low amounts of background contamination could have an impact (169, 170). These factors should be optimized for each type of specimen to ensure generation of unbiased microbiota profiles. In addition, standardization of these protocols is required to enable comparison of results obtained from different studies before translating them into the clinical setting.

16S rRNA gene profiling currently lacks accurate identification of bacteria at the species or strain level due to the lack of resolution of the small piece of conserved DNA used. As previously discussed, there are several ways to maximise the classification of 16S rRNA gene profiling, including sequencing of the full length amplified 16S rRNA gene. The latter has recently become possible by the release of third generation sequencing flatforms, such as the PacBio (Pacific Biosystems) and the MinION (Oxford Nanopore Techniques. The MinION platform collects and analyses sequence data in real-time, which can significantly shorten the time-to-result compared to other platforms. Nonetheless, the applicability of these

third-generation sequencing flatforms for 16S rRNA gene profiling remains to be confirmed due to the relatively high error rates (19, 20).

Alternative approaches are combining 16S rRNA gene profiling with species-specific qPCRs or employing WMS sequencing, which enables identification of bacteria and all other microorganisms (e.g. archaea, fungi and DNA viruses) present in a specimen to species or even strain level. More interestingly, this method has the potential to provide information about the abundance of genes involved in functional pathways present in a specimen (30). Identification of the functions of the microbiome in disease is currently hindered by a lack of functional characterisation of the vast majority of microbial genes that may be present in clinical specimens. Furthermore, technical challenges, ethical issues associated with sequencing of human DNA, higher costs, more complex data analysis as well as interpretation challenges comparable to that of 16S rRNA gene profiling, have to be solved before WMS sequencing is likely to be implemented in clinical microbiology (171-173). Until then, 16S rRNA gene profiling can fill the gap between traditional culture-based microbiological methods and WMS sequencing.

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CHAPTER 10

Summary
Nederlandse samenvatting
List of publications
Curriculum vitae
Dankwoord

SUMMARY

The human body is colonised by a vast number of archaea, bacteria, bacteriophages, fungi, protozoa and viruses, collectively referred to as the human microbiota. These microorganisms play a critical role in important physiological processes of the human body. Disturbance of the microbiota has been associated with a wide range of diseases and is therefore the focus of a growing number of research initiatives. The boost in microbiota research resulted from the development of the high-throughput sequencing platforms, which enabled easy detection of a theoretically unlimited number of bacteria based on the 16S ribosomal RNA (rRNA) gene. This gene is present in all bacteria and demonstrates considerable sequence diversity among different bacteria, making it a useful marker for identification of different bacteria. The most widely used approach is based on amplification and subsequently sequencing of the hypervariable regions of the 16S rRNA gene. The collection of obtained sequences is representative of the bacterial community as a whole in terms of membership (those present), and relative abundances, (how many 16S rRNA genes of a member is present compared to the total). This method can also be very valuable for clinical microbiology as it enables detection and identification of a theoretically unlimited number of bacteria present in a specimen and permits (semi)quantitative information about the composition of a microbial community. Although 16S rRNA gene profiling is a very straightforward method, its usefulness may be reduced due to its limited resolution to genus rather than the species level. In this thesis, we aimed to evaluate the clinical usefulness of 16S rRNA gene profiling. In addition, we explored whether clinically relevant cut-off values for interpretation of the sequencing data could be defined. Chapter 1 serves as a general introduction to the human microbiota and its association with disease. In addition, the different high-throughput sequencing methods for identification of bacteria are outlined. Finally, the potentially added value of 16S rRNA gene profiling for the clinical microbiology is explained.

In the first part of this thesis, we focused on using 16S rRNA gene profiling as a direct diagnostic tool for identification of clinically relevant bacteria. In routine clinical microbiology, standard identification of clinically relevant microorganisms from specimens is based on culture of bacteria 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. In **Chapter 2**, we questioned whether a stepwise approach using 16S rRNA gene profiling followed by species-specific real-time quantitative PCRs (qPCRs) and/or culture has the potential to be a more accurate and efficient diagnostic approach than the routine diagnostic approach based on culture. To this end, 62 sputum samples from patients with a suspected lower respiratory tract infection (LRTI) were analysed by the routine culture-based approach, 16S rRNA gene profiling and multiple species-specific qPCRs. We found that 16S rRNA gene

profiling identified 110 potentially pathogenic genera in sputum while 37 pathogens were found using a routine culture-based approach. This difference is caused by the fact that 16S rRNA gene profiling can detect all bacteria present in a specimen while culture is often more specific for a certain class of pathogenic species. Non-pathogenic species are not investigated by routine culture. Since non-pathogenic and pathogenic species can both colonise the respiratory tract without being involved in an infection, it is very important to provide a clinical interpretation of 16S rRNA gene profiling data in measures of relative abundance. This turned out to be a challenge. Furthermore, classification to the genus level is not always sufficient to identify the causative microorganism. Based on these observations, we concluded that 16S rRNA gene profiling provides a more complete characterisation of polymicrobial communities and can be used to identify potentially pathogenic genera in sputum, but only when combined with species-specific qPCR to achieve the needed resolution to the species level. Selective culture was added to the combination of methods to be performed to characterise phenotypic aspects such as the antibiotic susceptibility of a pathogen. Unfortunately, faster, better and cheaper technology for sequencing, which can be expected in the near future, are required before 16S rRNA gene profiling will be adopted by the routine clinical microbiology. Taken together, the stepwise approach using 16S rRNA gene profiling, species-specific qPCRs and culture has the potential to be used in clinical settings for the diagnosis of LRTIs in the near future when challenges of clinical data interpretation, turnaround time and cost of 16S rRNA gene profiling are overcome.

In the second part of this thesis, we focussed on using 16S rRNA gene profiling as an indirect tool for evaluation of diagnostic methods and therapies. In **Chapter 3**, we explored using 16S rRNA gene profiling as an alternative reference test to evaluate existing tests for the diagnosis of bacterial vaginosis (BV). To discriminate between a normal vaginal microbiota and BV, we used a previously reported cut-off value of 47% relative abundance of *Lactobacillus*. *Lactobacillus* dominated vaginal microbiota profiles (with ≥47% relative abundance of *Lactobacillus*) were categorised as normal vaginal microbiota and microbiota profiles with less *Lactobacillus* (<47% relative abundance) with mainly anaerobes as microbiota associated with BV. These definitions were used to categorise 115 vaginal swabs. For each swab, the result was compared with the outcome of five diagnostic methods: (i) a Gram stain scoring system based on the quantitative assessment of three different bacterial morphotypes, called the Nugent score; (ii) Amsel's clinical criteria; (iii) culture and (iv) two commercially available qPCRs. For the current gold standard (Nugent score), we found a specificity (true negative rate) of 100% and a sensitivity (true positive rate) of 64% for the diagnosis of BV using 16S rRNA gene profiling as the reference test. This means that the Nugent score is highly accurate for identification of women without BV but that it misses 36% of the women with BV. Evaluation of the discrepant results between 16S rRNA gene profiling and the Nugent score showed that the unresolved clinical importance of the intermediate

category was mainly responsible for the poor sensitivity of the current gold standard. For all five diagnostic methods, we found a specificity of at least 92%, suggesting that ≥47% relative abundance of *Lactobacillus* was highly accurate for identification of women without BV. However, the sensitivities of the five diagnostic methods ranged between 39% and 81%. Discrepancy analysis showed that at least two methods were in agreement with 16S rRNA gene profiling in 81% of the swabs categorised as microbiota associated with BV. The microbiota of the remaining swabs was characterised by a relatively high abundance of bacteria weakly associated with BV and/or not commonly found in the vagina. Based on these data, we consider 16S rRNA gene profiling as a good alternative for the current gold standard to evaluate BV diagnostic methods. Currently, the definition of <47% relative abundance of *Lactobacillus* and mainly anaerobes is sufficient to identify women with BV, but more research is required to identify the role of specific bacteria in the vaginal microbiota.

Next, we changed our focus from the evaluation of diagnostic methods to the evaluation of treatments. We discussed the usefulness of 16S rRNA gene profiling as a tool to determine the impact of treatment on polymicrobial communities. First, we focussed on the treatment of BV because up to 40% of the patients treated for BV return to their physician with persistent symptoms of abnormal vaginal discharge. Since the diagnosis of BV is challenging due to the subjective scoring of the Nugent test, the wide spectrum of anaerobes associated with BV and the limited number of bacteria that can be effectively cultivated or targeted by qPCR, misdiagnosis rather than treatment failure might be the cause of the persistent symptoms. In **Chapter 4**, we determined the best algorithm for the diagnosis of BV based on clinical symptoms and signs, bedside and/or laboratory test findings. To achieve this, tree-based classification analysis was performed on the clinical data and bedside test results obtained from 56 women with abnormal vaginal discharge. Laboratory tests were compared. We found that the best algorithm was to screen with two bedside tests, and if positive, to confirm with qPCR (sensitivity 94%; specificity 97%). Secondly, we performed retrospective analysis to determine whether implementation of the developed algorithm would have reduced the number of patients that returned to their physician with persistent symptoms. We found that 30% of the persistent symptoms were caused by misdiagnosis and another 30% by the occurrence of a different infection or treatment failure. For both analyses, 16S rRNA gene profiling and other reference tests were needed to diagnose BV as well as fungal and protozoan infections that may cause abnormal vaginal discharge. These data show that 16S rRNA gene profiling is not sufficient as stand alone test.

The next step is to understand why treatment failure occurs in some women with BV. In **Chapter 5**, we performed comprehensive analysis on the 16S rRNA gene profiling data obtained during two subsequent hospital visits from 21 women with clinically diagnosed BV. We observed two bacterial community types before and after antibiotic treatment with significantly different bacterial diversity. The community state types identified in women before treatment were driven by *Lactobacillus*, *Gardnerella* and *Atopobium* or by

Gardnerella, *Atopobium*, *Prevotella* and *Sneathia* while the community state types identified after treatment were either driven by *Lactobacillus* or by multiple bacteria. Unfortunately, we found no association between the community state types before or after treatment and the clinical outcome defined as: no, or persistent symptoms. It might be the case that treatment failure involves bacterial strains with high virulence potential, which cannot be differentiated from strains with low virulence potential based on the 16S rRNA gene. Taken together, the 16S rRNA gene profiling contributes to better understanding as to why women return to their physician with persistent complaints of abnormal vaginal discharge but lack resolution to discriminate between virulent and non-virulent bacterial strains.

In **Chapter 6**, we further explored the use of 16S rRNA gene profiling to determine the impact of treatment on the microbiota. We focussed on how 16S rRNA gene profiling can be applied in clinical trials to assess the effectiveness of new drugs in patients with atopic dermatitis (AD). For these clinical trials, the skin microbiota is an interesting biomarker since AD is associated with *S. aureus* colonisation and reduced microbial diversity. The microbiota composition of healthy skin may vary significantly between humans (inter-individual variation) and within a human over time (intra-individual variability) due to host and environmental factors, such as antibiotic exposure, hygiene and lifestyle. Importantly, skin affected by AD is likely to have larger inter- and intra-patient variability of the skin microbiota, implying the need for frequent sampling when evaluating the impact of treatment on the affected skin microbiota. Accordingly, we analysed 16S rRNA gene profiling, culture and qPCR data obtained from skin swabs collected weekly from 20 patients with mild to moderate AD. To measure the inter- and intra-patient variability over a period of 42 days, the standard deviation of the mean was calculated for microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration. For microbial diversity of lesional skin, we observed a high inter-patient variability (36-46% vs 16-28%) and a wide range of intra-patient variability (7-92% vs 4-29%) compared to unaffected skin. For the relative abundance of *Staphylococcus* spp. and *S. aureus* concentration of affected skin comparable results were found. In addition, we were able to define three patient groups with different microbiological phenotypes. We categorized these patients according to their microbial diversity of skin microbiota, the relative abundance of the genus *Staphylococcus*/*S. aureus* and microbiota and temporal variability. Groups I and II could be described as high relative abundance of *S. aureus*, low microbial diversity and either microbiologically stable, or unstable over time, respectively. In contrast, the affected skin microbiota of group III was closely related to their unaffected skin microbiota with a low relative abundance of *S. aureus* and high microbial diversity. Based on these observations, we concluded that microbiological phenotype stratification with 16S rRNA gene profiling and frequent sampling is required to analyse the microbiota in clinical AD trials.

In the third part of this thesis, we used 16S rRNA gene profiling as a research tool to study the link between microbiota and disease. Previously, we explored whether 16S rRNA gene profiling added value for clinical microbiology as a direct or indirect diagnostic tool. To address this, studies focussing on different diseases were performed, including BV and AD. Currently, microbiota research looking for a link between other diseases and the human microbiota is increasing exponentially. However, we believe more basic research is needed before microbiota findings can be safely and widely applied into the clinical setting. To illustrate this, we used 16S rRNA gene profiling as a research tool to study the link between the microbiota and two different medical conditions. In **Chapter 7**, we explored whether specific nasal and/or oropharyngeal microbiota profiles can be associated with increased age and RTIs in adults. To achieve this, 16S rRNA gene profiling was performed on nasal and oropharyngeal swabs of 152 controls and 152 patients with an upper or lower respiratory tract infection. We defined eight nasal and nine oropharyngeal microbiota clusters. Unfortunately, we were unable to clarify why the elderly are more susceptible to RTIs. Unexpectedly, we showed that nasal microbiota dominated by the genus *Moraxella* (presumably *Moraxella nonliquefaciens*) is associated with respiratory health in the elderly population. The nasal microbiota cluster dominated by the genus *Moraxella* was significantly more prevalent in the healthy elderly population ($p = 0.002$) compared to the healthy middle aged adults, and it was significantly less prevalent in the elderly with a LRTI ($p = 0.001$) compared to the healthy elderly population. However, some caution is necessary when translating this research based on 16S rRNA gene profiling into the clinic. We collected data at one timepoint. This means that we cannot distinguish whether *M. nonliquefaciens* was less prevalent in elderly patients due to its protective nature or that the infection changed the microbiota. Furthermore, fundamental research to explore the protective properties of *M. nonliquefaciens* is needed.

In **Chapter 8**, we explored whether the balanopreputial and urine microbiota are associated with a chronic inflammatory, scarring dermatosis associated with penile cancer, called male genital lichen sclerosus (MGLSc). Accordingly, 16S rRNA gene profiling was performed on balanopreputial swabs and urine of 40 controls and 40 men with MGLSc. For the balanopreputial sac, we observed a difference in microbiota profiles between the men with MGLSc and controls. The relative abundance of the genus *Finegoldia* was decreased in men with MGLSc (median relative abundance of 9% vs 28%) while the relative abundance of the genus *Prevotella* was increased (median relative abundance of 20% vs 4%). Both the prevalence (50% vs 15%) and relative abundance (median relative abundance of 4% vs 0%) for the genus *Fusobacterium* were increased in men with MGLSc. *Fusobacterium* spp., particularly *Fusobacterium nucleatum*, have been associated with inflammatory diseases such as periodontitis and inflammatory bowel disease and some cancers such as colorectal and

oral squamous cell carcinoma. Longitudinal data is lacking to establish whether dysbiosis of the balanopreputial sac microbiota is the cause or consequence of the disease. Furthermore, involvement of balanopreputial sac dysbiosis in the aetiology and pathogenesis of MGLSc, with a specific role for *F. nucleatum*, should be confirmed by reproduction of the disease *In vitro* or in animal models. Thereafter, interventional research can be performed to develop treatment for specific diseases.

Finally, in **Chapter 9** we evaluated the results of the studies described in this thesis and discussed their implications for future research. In this final chapter, we highlighted the clinical application for which 16S rRNA gene profiling should be considered and the corresponding data interpretation challenges. Furthermore, we speculated about the usefulness of whole metagenomic shotgun sequencing for clinical microbiology compared to 16S rRNA gene profiling. The clinical value of 16S rRNA gene profiling will probably become clear in the forthcoming years.

NEDERLANDSE SAMENVATTING

Alle oppervlaktes van het menselijk lichaam zijn bedekt met een enorm aantal micro-organismen, waaronder bacteriën, schimmels en virussen. De samenstelling van deze microbiële gemeenschap wordt de 'microbiota' genoemd. Deze microbiële gemeenschappen zijn biochemisch van groot belang voor het menselijk lichaam. Om deze reden wordt de microbiota van verschillende lichaamslocaties intensief bestudeerd en probeert men de invloed van de microbiota op onze gezondheid vast te stellen. De bacteriële samenstelling van de microbiota wordt veelal in kaart gebracht door het 16S ribosomale RNA (rRNA) gen te onderzoeken. Het 16S rRNA gen heeft de eigenschap dat het aanwezig is in alle bacteriën. Tevens bevat dit gen naast geconserveerde DNA-gebieden ook variabele DNA-gebieden die uniek zijn per bacteriegeslacht en dus gebruikt kunnen worden voor bacteriële identificatie. Daarvoor worden één of meerdere variabele DNA-gebieden eerst vermeerderd met behulp van PCR-technieken om vervolgens de volgorde van de bouwstenen van het DNA te bepalen middels high-througput sequencing technieken. De verkregen informatie kan gebruikt worden om alle bacteriegeslachten aanwezig in een klinisch monster te identificeren en de relatieve verhoudingen te bepalen d.w.z. het bepalen van het aantal 16S rRNA genen van een specifieke bacteriegeslacht ten opzichte van het totaal aantal 16S rRNA genen aanwezig in een klinisch monster. Deze werkwijze, bekend als '16S rRNA gene profiling', zou ook waardevol kunnen zijn voor de klinische microbiologie omdat het men in staat stelt om een theoretisch onbeperkt aantal bacteriegeslachten in een klinisch monster te identificeren zonder de afzonderlijke bacteriën te hoeven kweken. Dit laatste is een belangrijk gegeven aangezien de meeste bacteriën lastig te kweken zijn in een laboratorium. De bruikbaarheid van 16S rRNA gene profiling zou beperkt kunnen zijn doordat verschillende bacteriesoorten (species level) onder eenzelfde bacteriegeslacht (genus level) vallen. Het doel van het onderzoek beschreven in dit proefschrift is dan ook het evalueren van de klinische bruikbaarheid van 16S rRNA gene profiling. **Hoofdstuk 1** fungeert als een algemene inleiding waarin de term microbiota en zijn associatie met ziekten wordt uitgelegd. Daarnaast worden de verschillende high-throughput sequencing methoden voor identificatie van bacteriën beschreven. Tenslotte wordt de mogelijke waarde van 16S rRNA gene profiling voor de klinische microbiologie besproken.

In het eerste deel van dit proefschrift onderzochten we de bruikbaarheid van 16S rRNA gene profiling voor de identificatie van ziekteverwekkende bacteriën. In de routine klinische microbiologie, identificatie van ziekteverwekkers wordt standaard gedaan door het opkweken van bacteriën uit een klinisch monster, waarna de bacteriesoort en de antibioticagevoeligheid bepaald kan worden. In **Hoofdstuk 2** hebben we onderzocht of een combinatie van 16S rRNA gene profiling met andere technieken potentie heeft om gebruikt te worden in de routine diagnostiek. Hiervoor hebben we 62 sputum monsters

van patiënten met een lagere luchtweginfectie geanalyseerd. Met 16S rRNA gene profiling vonden we 110 mogelijke ziekteverwekkers. Door de routine klinische microbiologie waren er 37 ziekteverwekkers gevonden. Dit verschil wordt veroorzaakt doordat 16S rRNA gene profiling geen onderscheid kan maken tussen ziekteverwekkende en onschuldige bacteriesoorten binnen een bacteriegeslachten, terwijl de routine klinische microbiologie zich focust op een kleine groep ziekteverwekkers. Vandaar dat het belangrijk is om te bepalen welke relatieve hoeveelheid van een bacteriegeslacht klinisch relevant is, d.w.z. wijzen op betrokkenheid bij een infectie. Dit bleek een uitdaging. Hierop hebben we gebaseerd dat 16S rRNA gene profiling een beter overzicht geeft van de bacteriële samenstelling van een sputum monster en dat het potentie heeft om ziekteverwekkers te identificeren maar alleen in combinatie met een test die onderscheid maakt tussen ziekteverwekkende en onschuldige bacteriesoorten. Daarnaast adviseren wij om kweek toe te voegen aan deze combinatie van testen om, wanneer wenselijk, de antibioticagevoeligheid van een ziekteverwekker te kunnen bepalen. Wel zullen eerst uitdagingen zoals klinische relevantie van relatieve hoeveelheden van verschillende bacteriegeslachten, tijdsduur van sample afname tot uitslag met 16S rRNA gene profiling en kosten overwonnen dienen te worden.

In het tweede deel van dit proefschrift hebben we onderzocht of 16S rRNA gene profiling voor de evaluatie van diagnostisch methoden en behandelingen gebruikt zou kunnen worden. Hiervoor hebben we ons eerst gericht op het vaginale microbiota. Onder gezonde vaginale microbiota verstaan wij dominantie van het bacteriegeslacht *Lactobacillus*. Wanneer vrouwen afwijkende vaginale afscheiding hebben, is het mogelijk dat de balans van de vaginale microbiota is verstoord. Bij een verschuiving van vaginale microbiota gedomineerd door *Lactobacillus* naar een meer gevarieerd microbiota met vooral bacteriën die zonder zuurstof kunnen leven (anaerobe bacteriën), spreken we van bacteriële vaginose (BV). In **Hoofdstuk 3** hebben we onderzocht of 16S rRNA gene profiling als een alternatieve referentie test voor het evalueren van bestaande diagnostische testen gebruikt zou kunnen worden. Voor dit doel werden voor 115 vaginale swabs/vrouwen de resultaten van vijf verschillende diagnostische testen vergeleken met 16S rRNA gene profiling. Voor 16S rRNA gene profiling hebben we daarbij gebruik gemaakt van een gepubliceerde afkapwaarde voor de relatieve hoeveelheid van *Lactobacillus* om onderscheid te maken tussen gezonde vaginale microbiota en BV. Iedere diagnostische test was in minimaal 92% van de swabs het eens met de BV negatieve uitslag van 16S rRNA gene profiling. Dit suggereert dat de gebruikte afkapwaarde voor *Lactobacillus* erg accuraat is om BV negatieve vrouwen mee te identificeren. Van de swabs die BV positief waren met 16S rRNA gene profiling was iedere diagnostische test het eens in 39%-82% van de swabs. De diagnostische methodes waren het vaak niet met elkaar eens wat suggereert dat het lastig is om vrouwen met BV te identificeren. Deze onenigheid lijkt te worden veroorzaakt door de onduidelijke symptomen, de hoge variatie aan bacteriën die met BV worden geassocieerd en de beperkte aantal

bacteriën die gedetecteerd kunnen worden met een diagnostische test. Hier heeft 16S rRNA gene profiling geen last van. Hierop hebben we gebaseerd dat 16S rRNA gene profiling een goede alternatieve referentie test zou zijn voor het evalueren van diagnostische testen.

Vervolgens hebben we onze aandacht verschoven van evaluatie van diagnostische testen naar de evaluatie van behandelingen. Van de vrouwen die behandeld worden voor BV, keer 40% terug bij de arts met dezelfde klachten als voor de behandeling. In **Hoofstuk 4** hebben we eerst bepaald of deze blijvende klachten het resultaat waren van een falende behandeling of dat de vrouwen verkeerd waren gediagnostiseerd en daardoor een verkeerde behandeling hadden gekregen. Hiervoor werd de vastgestelde diagnose van de arts vergeleken met de test resultaten van 16S rRNA gene profiling en diagnostische testen voor vaginale schimmelinfecties en seksuele overdraagbare aandoeningen. Van de 27 vrouwen die terugkeerde bij de arts met dezelfde klachten, bleek dat 30% een verkeerde diagnose had gehad en dat in een andere 30% van de vrouwen de behandeling niet werkte of ze hadden een andere vaginale infectie opgelopen na de behandeling. Deze data toonde aan dat 16S rRNA gene profiling niet altijd voldoende is als onafhankelijke test, omdat het alleen informatie geeft over bacteriën en niet over de aanwezigheid van andere organismen. De volgende stap was om te begrijpen waarom behandeling van BV faalt voor sommige vrouwen. In **Hoofdstuk 5** hebben we uitgebreide analyses uitgevoerd op de 16S rRNA gene profiling data van 21 vrouwen met BV waarvan 9 klachten hielden na de behandeling. Voor en na behandeling konden we twee verschillende microbiota onderscheiden. Voor behandeling werden de microbiota gekarakteriseerd door de aanwezigheid van de bacteriegeslachten *Lactobacillus*, *Gardnerella* en *Atopobium* of door *Gardnerella*, *Atopobium*, *Prevotella* en *Sneathia*. Na behandeling werd de microbiota gekarakteriseerd door de dominantie van *Lactobacillus* of de aanwezigheid van verschillende bacteriën. Helaas vonden we geen associatie tussen de microbiota samenstelling voor of na behandeling en blijvende klachten. Het is mogelijk dat specifieke bacteriestammen met hoog ziekmakend vermogen betrokken zijn bij het falen van de behandeling. Deze kunnen helaas met 16S rRNA gene profiling niet onderscheiden worden van bacteriestammen met een lager ziekmakend vermogen. Samengevat, 16S rRNA gene profiling is een handige tool voor het beter begrijpen waarom sommige vrouwen terugkeren bij de arts met dezelfde klachten als voor de behandeling van BV, maar het niet kunnen onderscheiden van bacteriestammen met hoog en laag ziekmakend vermogen vormt een tekortkoming van deze methode.

In **Hoofdstuk 6** hebben we verder gekeken naar de bruikbaarheid van 16S rRNA gene profiling voor het bepalen van de impact van een behandeling op de microbiota. Deze keer hebben we onderzocht hoe 16S rRNA gene profiling toegepast zou kunnen worden in klinische studies voor het evalueren van de effectiviteit van nieuwe medicijnen in patiënten met atopisch dermatitis (AD) oftewel atopisch eczema. Voor deze klinische studies is de huid microbiota interessant, omdat AD geassocieerd is met kolonisatie van de huid met de bacterie *Staphylococcus aureus* en verminderde microbiota diversiteit. De microbiota

compositie van gezonde huid kan erg verschillen tussen individuelen (inter-individuele variatie) en over tijd bij eenzelfde individu (intra-individuele variatie) door gastheer- en omgevingsfactoren, zoals antibioticagebruik, hygiëne en levensstijl. Het is waarschijnlijk dat de aangedane huid van patiënten met AD een nog grotere inter- en intra-individuele variatie vertoont. Dit zou betekenen dat het nodig is om regelmatig huid swabs af te nemen voor het evalueren van de impact van een behandeling op de huid microbiota. Om deze reden hebben we de inter- en intra-individuele variatie van de huid microbiota bepaald bij 20 patiënten met AD bij wie wekelijks, over een periode van 42 weken, huid swabs waren afgenomen. In vergelijking met de niet aangedane huid vonden we een erge hoge variatie in microbiota diversiteit tussen patiënten en een erg variabele microbiota diversiteit over tijd. Vergelijkbare resultaten vonden we voor de relatieve hoeveelheid van het bacteriegeslacht *Staphylococcus* en concentratie van het bacteriesoort *Staphylococcus aureus*. Daarnaast konden we de personen in drie groepen indelen op basis van de microbiota diversiteit, hoeveelheid *Staphylococcus aureus* en microbiota stabiliteit over tijd. Deze resultaten bevestigen dat het regelmatig afnemen van huid swabs nodig is om goede conclusies te kunnen trekken over de impact van een behandeling op de aangedane huid microbiota van patiënten met AD.

In het derde deel van dit proefschrift hebben we 16S rRNA gene profiling als onderzoeksmethode gebruikt om de link tussen microbiota en ziektes te onderzoeken. Momenteel wordt dit type onderzoek veel uitgevoerd, maar wij geloven dat meer fundamenteel onderzoek nodig is voordat microbiota studies vertaald kunnen worden naar de kliniek. Om dit te illustreren hebben we in **Hoofdstuk 7** 16S rRNA gene profiling gebruikt om te onderzoeken of specifieke neus en/of keel microbiota profielen geassocieerd zijn met hogere leeftijd en luchtweginfecties. Hiervoor hebben we microbiota data van 152 controlepersonen en 152 patiënten met een bovenste of onderste luchtweginfectie geanalyseerd. In totaal hebben we 8 neus en 9 keel microbiota profielen kunnen onderscheiden. Helaas konden we niet aantonen waarom ouderen gevoeliger zijn voor luchtweginfecties ten opzichte van jongvolwassenen. Verrassend genoeg vonden we wel aanwijzingen voor een associatie tussen gezondheid en neus microbiota gedomineerd door de bacteriesoort *Moraxella nonliquefaciens* in de oudere populatie. Op basis van deze resultaten zou je kunnen zeggen dat de oudere populatie voordeel heeft bij kolonisatie met *Moraxella nonliquefaciens*. Toch is er voorzichtigheid geboden met het vertalen van dit onderzoek naar de kliniek. De resultaten zijn gebaseerd op een momentopname en om aan te kunnen tonen dat *Moraxella nonliquefaciens* echt geassocieerd is met gezondheid zou een grote populatie mensen over tijd gevolgd moeten worden. Daarnaast is fundamenteel onderzoek nodig om de beschermende eigenschappen van *Moraxella nonliquefaciens* te onderzoeken voordat we *Moraxella nonliquefaciens* gaan toedienen bij ouderen als probiotica. Een tweede studie die illustreert dat we voorzichtig moeten zijn met het direct

vertalen van microbiota onderzoek naar de kliniek is beschreven in **Hoofdstuk 8**. Hier hebben we onderzocht of er mogelijke associatie is tussen de penis en urine microbiota en een chronische inflammatoire, littekendermatose geassocieerd met peniskanker oftewel male genital lichen sclerosus (MGLSc). Voor dit doel hebben we microbiota data geanalyseerd van 40 controlepersonen en 40 mannen met MGLSc. We vonden een aantal verschillen tussen de penis microbiota van gezonde mannen en mannen met MGLSc. Een verschil daarvan was dat het de prevalentie en de relatieve hoeveelheid van het bacteriegeslacht *Fusobacterium* verhoogd leek te zijn in mannen met MGLSc ten opzichte van de controle populatie. Dit was met name interessant omdat de bacteriesoort *Fusobacterium nucleatum* geassocieerd wordt met verschillende inflammatoire ziektes en kanker. Bij het trekken van conclusies speelt hier opnieuw monster afname een rol. De monsters zijn verzameld op een enkel moment in tijd waardoor we niet kunnen bepalen of de verschillen in microbiota de oorzaak ofwel een gevolg van de ziekte zijn. Daarnaast zou de rol van de penis microbiota in het ontstaan van MGLSc bevestigd moeten worden in een diermodel. Daarna zou pas interventie onderzoek uitgevoerd kunnen worden om nieuwe behandelingen voor patiënten met MGLSc te kunnen onderzoeken. Kortom, resultaten van microbiota studies kunnen niet direct veilig en breed vertaald worden naar de kliniek.

In het laatste deel van dit proefschrift, **Hoofdstuk 9**, evalueren we de resultaten van de onderzoeken beschreven in dit proefschrift en bediscussiëren we toekomstige onderzoeken.

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CURRICULUM VITAE

Ellen Hendrika Adriana van den Munckhof werd geboren op 11 maart 1988 te Delft. Zij groeide op in Nootdorp met haar ouders en jongere zussen Ilse en Susan. Haar vwo-diploma behaalde zij in 2006 aan het Huygens college te Voorburg. Vervolgens begon zij aan de bachelor Biomedische Wetenschappen aan de Universiteit van Leiden. Tijdens haar bachelor stage deed zij onderzoek naar tumorimmunologie onder leiding van Ir. Margit H. Lampen binnen de afdeling Klinische Oncologie van het Leids Universitair Medisch Centrum (LUMC). In 2009 behaalde zij haar bachelorsdiploma en begon zij aan de master Biomedische Wetenschappen. In het kader van deze master deed zij twee onderzoeksstages: eerst begon ze een onderzoek naar het remmen van Nidovirus RNA-synthese onder supervisie van Dr. Martijn J. van Hemert binnen de afdeling Medisch Microbiologie van het LUMC. Vervolgens heeft zij voor Crucell Holland B.V een real-time PCR opgezet voor het kwantificeren van transgene transcriptie van Ad26/Ad35 adenovector gebaseerde vaccines onder supervisie van Saskia Crowe en Joost Donkers. In augustus 2011 behaalde zij haar masterdiploma Biomedische Wetenschappen. Hierna ging zij aan de slag als junior scientist bij DDL Diagnostic Laboratory. In deze functie voerde zij haar promotieonderzoek uit onder supervisie van prof. dr. Ed J. Kuijper, dr. Cornelis W. Knetsch en dr. Maurine van Hall-Leverstein, welk resulteerde in deze thesis.

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