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**).

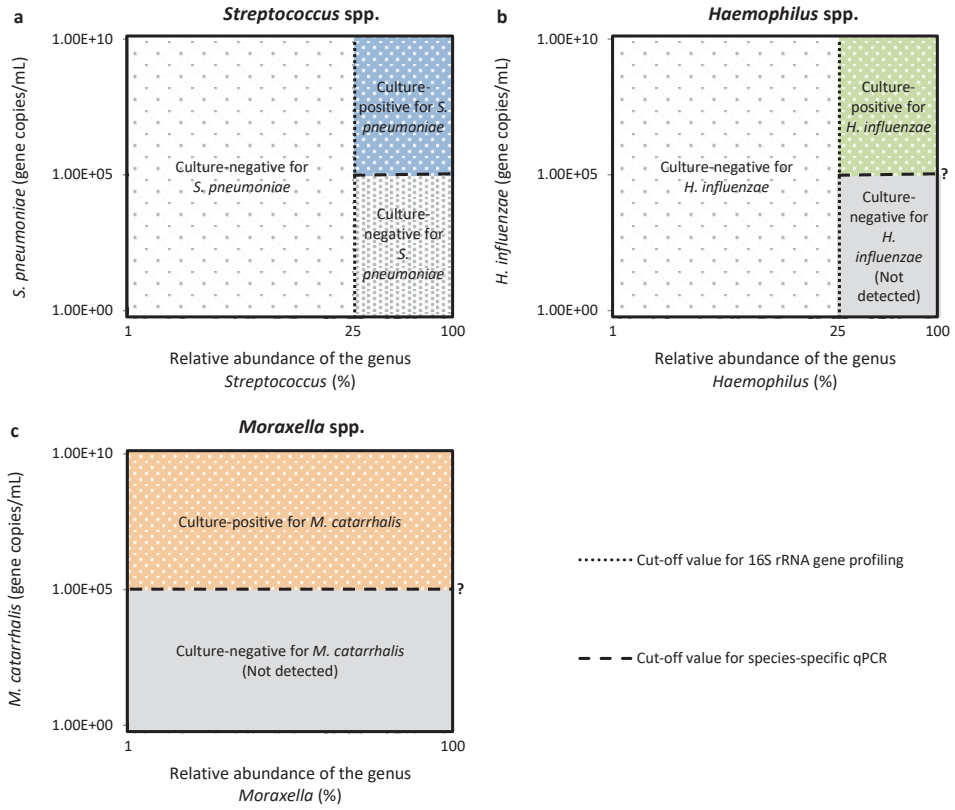

Figure 1. Clinical data interpretation of 16S rRNA gene profiling at the genus level (x-axis) and species-specific quantitative real-time PCRs (qPCRs; y-axis) of sputum is challenging as illustrated for (**a**) *Streptococcus* (**b**) *Haemophilus* and (**c**) *Moraxella* spp. For identification of *Streptococcus 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%).

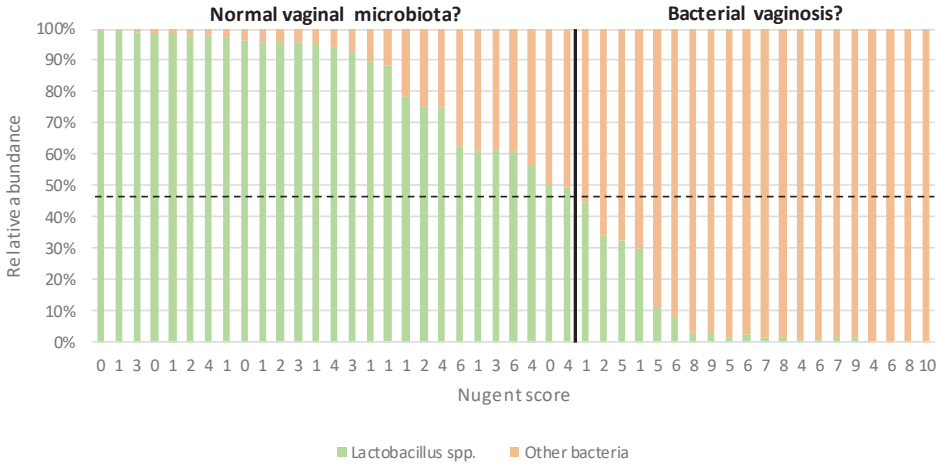

Figure 2. Defining a cut-off value (dotted line) for the genus *Lactobacillus* in measures of relative abundances (y-axis) to discriminate between normal vaginal microbiota and bacterial vaginosis is challenging as illustrated by the Nugent score (x-axis). A Nugent score of 0 to 3 is consistent with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

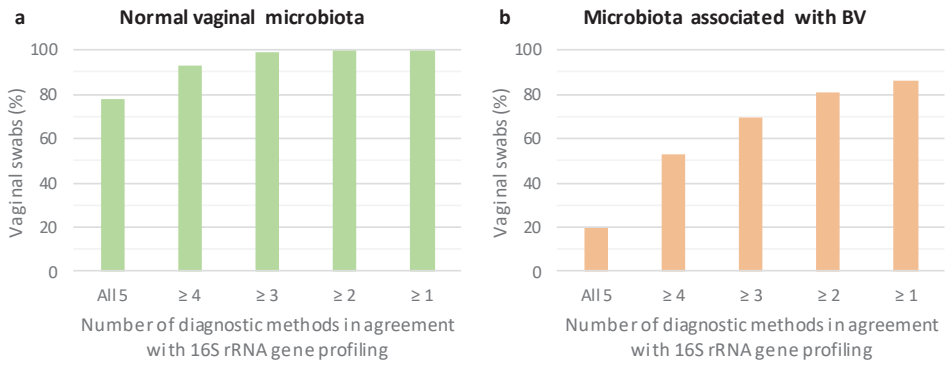

Figure 3. Defining (**a**) normal vaginal microbiota is less complicated than (**b**) microbiota associated with bacterial vaginosis (BV) as illustrated by the outcome comparison of 16S rRNA gene profiling and five diagnostic methods (Nugent score, Amsel's clinical criteria, culture and two CE-IVD quantitative 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.

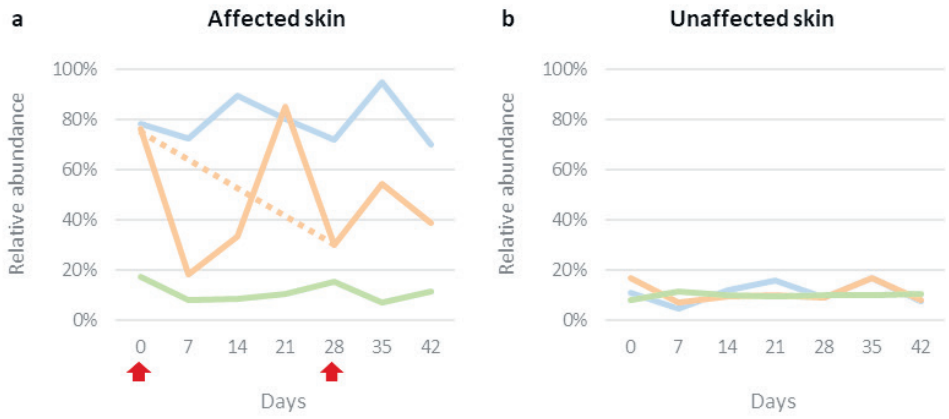

Figure 4. Limited sampling is insufficient to capture the full extent of skin microbiota variability in patients with atopic dermatitis as illustrated by the relative abundance of the genus *Staphylococcus* (x-axis)over time (y-axis) for (**a**) affected and (**b**) unaffected skin of patients with microbiological 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

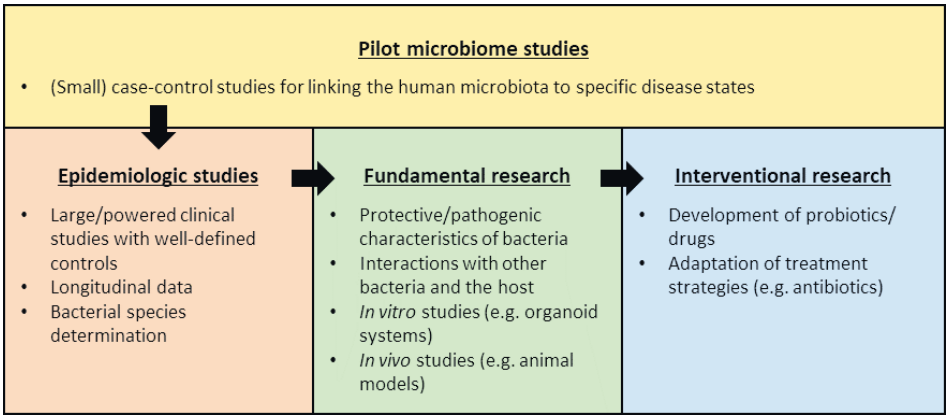

Figure 5. Translation science

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.

REFERENCES

1. van Prehn J, van Veen SQ, Schelfaut JJ, and Wessels E. MALDI-TOF mass spectrometry for differentiation between Streptococcus pneumoniae and Streptococcus pseudopneumoniae. *Diagn Microbiol Infect Dis*. 2016.
2. Leber AL, ed. *Clinical microbiology procedures handbook*. Fourth ed. ASM Books. Vol. 1-3. 2016, ASM Press: Washington, DC.
3. Edin A, Granholm S, Koskiniemi S, Allard A, Sjostedt A, and Johansson A. Development and laboratory evaluation of a real-time PCR assay for detecting viruses and bacteria of relevance for community-acquired pneumonia. *J Mol Diagn*. 2015;17(3):315-24.
4. Human Microbiome Project C. Structure, function and diversity of the healthy human microbiome. *Nature*. 2012;486(7402):207-14.
5. Dickson RP and Huffnagle GB. The Lung Microbiome: New Principles for Respiratory Bacteriology in Health and Disease. *PLoS Pathog*. 2015;11(7):e1004923.
6. Hang J, Zavaljevski N, Yang Y, Desai V, Ruck RC, Macareo LR, Jarman RG, Reifman J, Kuschner RA, and Keiser PB. Composition and variation of respiratory microbiota in healthy military personnel. *PLoS One*. 2017;12(12):e0188461.
7. Garcia LS and Isenberg HD, eds. *Clinical microbiology procedures handbook*. third ed. ASM Books. Vol. 1-3. 2010, ASM Press: Washington DC.
8. Huffnagle GB, Dickson RP, and Lukacs NW. The respiratory tract microbiome and lung inflammation: a two-way street. *Mucosal Immunol*. 2017;10(2):299-306.
9. Murphy TF, Brauer AL, Grant BJ, and Sethi S. Moraxella catarrhalis in chronic obstructive pulmonary disease: burden of disease and immune response. *Am J Respir Crit Care Med*. 2005;172(2):195-9.
10. Quainoo S, Coolen JPM, van Hijum S, Huynen MA, Melchers WJG, van Schaik W, and Wertheim HFL. Whole-Genome Sequencing of Bacterial Pathogens: the Future of Nosocomial Outbreak Analysis. *Clin Microbiol Rev*. 2017;30(4):1015-1063.
11. Zhou K, Lokate M, Deurenberg RH, Tepper M, Arends JP, Raangs EG, Lo-Ten-Foe J, Grundmann H, Rossen JW, and Friedrich AW. Use of whole-genome sequencing to trace, control and characterize the regional expansion of extended-spectrum beta-lactamase producing ST15 Klebsiella pneumoniae. *Sci Rep*. 2016;6:20840.
12. Zhou K, Ferdous M, de Boer RF, Kooistra-Smid AM, Grundmann H, Friedrich AW, and Rossen JW. The mosaic genome structure and phylogeny of Shiga toxin-producing Escherichia coli O104:H4 is driven by short-term adaptation. *Clin Microbiol Infect*. 2015;21(5):468 e7-18.
13. Ferdous M, Zhou K, de Boer RF, Friedrich AW, Kooistra-Smid AM, and Rossen JW. Comprehensive Characterization of Escherichia coli O104:H4 Isolated from Patients in the Netherlands. *Front Microbiol*. 2015;6:1348.
14. Campos ACC, Andrade NL, Ferdous M, Chlebowicz MA, Santos CC, Correal JCD, Lo Ten Foe JR, Rosa ACP, Damasco PV, Friedrich AW, and Rossen JWA. Comprehensive Molecular Characterization of Escherichia coli Isolates from Urine Samples of Hospitalized Patients in Rio de Janeiro, Brazil. *Front Microbiol*. 2018;9:243.

15. Besser JM, Carleton HA, Trees E, Stroika SG, Hise K, Wise M, and Gerner-Smidt P. Interpretation of Whole-Genome Sequencing for Enteric Disease Surveillance and Outbreak Investigation. *Foodborne Pathog Dis*. 2019;16(7):504-512.
16. Gerner-Smidt P, Besser J, Concepcion-Acevedo J, Folster JP, Huffman J, Joseph LA, Kucerova Z, Nichols MC, Schwensohn CA, and Tolar B. Whole Genome Sequencing: Bridging One-Health Surveillance of Foodborne Diseases. *Front Public Health*. 2019;7:172.
17. Balvociute M and Huson DH. SILVA, RDP, Greengenes, NCBI and OTT - how do these taxonomies compare? *BMC Genomics*. 2017;18(Suppl 2):114.
18. Kaehler BD, Bokulich NA, McDonald D, Knight R, Caporaso JG, and Huttley GA. Species abundance information improves sequence taxonomy classification accuracy. *Nat Commun*. 2019;10(1):4643.
19. Johnson JS, Spakowicz DJ, Hong BY, Petersen LM, Demkowicz P, Chen L, Leopold SR, Hanson BM, Agresta HO, Gerstein M, Sodergren E, and Weinstock GM. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nat Commun*. 2019;10(1):5029.
20. Kerkhof LJ, Dillon KP, Haggblom MM, and McGuinness LR. Profiling bacterial communities by MinION sequencing of ribosomal operons. *Microbiome*. 2017;5(1):116.
21. Janda JM and Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol*. 2007;45(9):2761-4.
22. Pei AY, Oberdorf WE, Nossa CW, Agarwal A, Chokshi P, Gerz EA, Jin Z, Lee P, Yang L, Poles M, Brown SM, Sotero S, Desantis T, Brodie E, Nelson K, and Pei Z. Diversity of 16S rRNA genes within individual prokaryotic genomes. *Appl Environ Microbiol*. 2010;76(12):3886-97.
23. Stralin K, Herrmann B, Abdeldaim G, Olcen P, Holmberg H, and Molling P. Comparison of sputum and nasopharyngeal aspirate samples and of the PCR gene targets lytA and Spn9802 for quantitative PCR for rapid detection of pneumococcal pneumonia. *J Clin Microbiol*. 2014;52(1):83-9.
24. Murphy TF and Parameswaran GI. Moraxella catarrhalis, a human respiratory tract pathogen. *Clin Infect Dis*. 2009;49(1):124-31.
25. Byrnes MC, Irwin E, Reicks P, and Brodsky I. Prospective, protocolized study evaluating effects of antibiotics on sputum culture results in injured patients. *Surg Infect (Larchmt)*. 2013;14(1):24-9.
26. Driscoll AJ, Deloria Knoll M, Hammitt LL, Baggett HC, Brooks WA, Feikin DR, Kotloff KL, Levine OS, Madhi SA, O'Brien KL, Scott JAG, Thea DM, Howie SRC, Adrian PV, Ahmed D, DeLuca AN, Ebruke BE, Gitahi C, Higdon MM, Kaewpan A, Karani A, Karron RA, Mazumder R, McLellan J, Moore DP, Mwananyanda L, Park DE, Prosperi C, Rhodes J, Saifullah M, Seidenberg P, Sow SO, Tamboura B, Zeger SL, Murdoch DR, and Group PS. The Effect of Antibiotic Exposure and Specimen Volume on the Detection of Bacterial Pathogens in Children With Pneumonia. *Clin Infect Dis*. 2017;64(suppl_3):S368-S377.
27. Chan M, Koo SH, Jiang B, Lim PQ, and Tan TY. Comparison of the Biofire FilmArray Respiratory Panel, Seegene AnyplexII RV16, and Argene for the detection of respiratory viruses. *J Clin Virol*. 2018;106:13-17.
28. Diaz-Decaro JD, Green NM, and Godwin HA. Critical evaluation of FDA-approved respiratory multiplex assays for public health surveillance. *Expert Rev Mol Diagn*. 2018;18(7):631-643.
29. Huang HS, Tsai CL, Chang J, Hsu TC, Lin S, and Lee CC. Multiplex PCR system for the rapid diagnosis of respiratory virus infection: systematic review and meta-analysis. *Clin Microbiol Infect*. 2018;24(10):1055-1063.
30. Quince C, Walker AW, Simpson JT, Loman NJ, and Segata N. Shotgun metagenomics, from sampling

to analysis. *Nat Biotechnol*. 2017;35(9):833-844.

31. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, Hu Y, Tao ZW, Tian JH, Pei YY, Yuan ML, Zhang YL, Dai FH, Liu Y, Wang QM, Zheng JJ, Xu L, Holmes EC, and Zhang YZ. A new coronavirus associated with human respiratory disease in China. *Nature*. 2020;579(7798):265-269.
32. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, Wang W, Song H, Huang B, Zhu N, Bi Y, Ma X, Zhan F, Wang L, Hu T, Zhou H, Hu Z, Zhou W, Zhao L, Chen J, Meng Y, Wang J, Lin Y, Yuan J, Xie Z, Ma J, Liu WJ, Wang D, Xu W, Holmes EC, Gao GF, Wu G, Chen W, Shi W, and Tan W. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet*. 2020;395(10224):565-574.
33. WHO. Laboratorium testing for coronavirus disease (COVID-19) in suspected human cases. 2020.
34. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, Bleicker T, Brunink S, Schneider J, Schmidt ML, Mulders DG, Haagmans BL, van der Veer B, van den Brink S, Wijsman L, Goderski G, Romette JL, Ellis J, Zambon M, Peiris M, Goossens H, Reusken C, Koopmans MP, and Drosten C. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill*. 2020;25(3).
35. Kupferschmidt K and Cohen J. Race to find COVID-19 treatments accelerates. *Science*. 2020;367(6485):1412-1413.
36. Callaway E. Scores of coronavirus vaccines are in competition - how will scientists choose the best? *Nature*. 2020.
37. Callaway E. The race for coronavirus vaccines: a graphical guide. *Nature*. 2020;580(7805):576-577.
38. Zastrow M. Open science takes on the coronavirus pandemic. *Nature*. 2020.
39. Ellington MJ, Ekelund O, Aarestrup FM, Canton R, Doumith M, Giske C, Grundman H, Hasman H, Holden MTG, Hopkins KL, Iredell J, Kahlmeter G, Koser CU, MacGowan A, Mevius D, Mulvey M, Naas T, Peto T, Rolain JM, Samuelsen O, and Woodford N. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. *Clin Microbiol Infect*. 2017;23(1):2-22.
40. Jajou R, van der Laan T, de Zwaan R, Kamst M, Mulder A, de Neeling A, Anthony R, and van Soolingen D. WGS more accurately predicts susceptibility of Mycobacterium tuberculosis to first-line drugs than phenotypic testing. *J Antimicrob Chemother*. 2019;74(9):2605-2616.
41. Malla MA, Dubey A, Kumar A, Yadav S, Hashem A, and Abd Allah EF. Exploring the Human Microbiome: The Potential Future Role of Next-Generation Sequencing in Disease Diagnosis and Treatment. *Front Immunol*. 2018;9:2868.
42. https://www.illumina.com/systems/sequencing-platforms.html.
43. Rossen JWA, Friedrich AW, Moran-Gilad J, Genomic ESGf, and Molecular D. Practical issues in implementing whole-genome-sequencing in routine diagnostic microbiology. *Clin Microbiol Infect*. 2018;24(4):355-360.
44. Sabat AJ, van Zanten E, Akkerboom V, Wisselink G, van Slochteren K, de Boer RF, Hendrix R, Friedrich AW, Rossen JWA, and Kooistra-Smid A. Targeted next-generation sequencing of the 16S-23S rRNA region for culture-independent bacterial identification - increased discrimination of closely related species. *Sci Rep*. 2017;7(1):3434.
45. Schuurman T, de Boer RF, Kooistra-Smid AM, and van Zwet AA. Prospective study of use of PCR amplification and sequencing of 16S ribosomal DNA from cerebrospinal fluid for diagnosis of bacterial meningitis in a clinical setting. *J Clin Microbiol*. 2004;42(2):734-40.

46. Lamont RF, Sobel JD, Akins RA, Hassan SS, Chaiworapongsa T, Kusanovic JP, and Romero R. The vaginal microbiome: new information about genital tract flora using molecular based techniques. *BJOG*. 2011;118(5):533-49.
47. Sherrard J, Wilson J, Donders G, Mendling W, and Jensen JS. 2018 European (IUSTI/WHO) International Union against sexually transmitted infections (IUSTI) World Health Organisation (WHO) guideline on the management of vaginal discharge. *Int J STD AIDS*. 2018:956462418785451.
48. Amsel R, Totten PA, Spiegel CA, Chen KC, Eschenbach D, and Holmes KK. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *Am J Med*. 1983;74(1):14-22.
49. Nugent RP, Krohn MA, and Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol*. 1991;29(2):297-301.
50. Dhiman N and Yourshaw C. Diagnostic Evaluation of a Multiplex Quantitative Real-Time PCR Assay for Bacterial Vaginosis. *Journal of Womens Health Care*. 2016;05(01):3.
51. Gaydos CA, Beqaj S, Schwebke JR, Lebed J, Smith B, Davis TE, Fife KH, Nyirjesy P, Spurrell T, Furgerson D, Coleman J, Paradis S, and Cooper CK. Clinical Validation of a Test for the Diagnosis of Vaginitis. *Obstet Gynecol*. 2017;130(1):181-189.
52. Gutman RE, Peipert JF, Weitzen S, and Blume J. Evaluation of clinical methods for diagnosing bacterial vaginosis. *Obstet Gynecol*. 2005;105(3):551-6.
53. Mohammadzadeh F, Dolatian M, Jorjani M, and Alavi Majd H. Diagnostic value of Amsel's clinical criteria for diagnosis of bacterial vaginosis. *Glob J Health Sci*. 2014;7(3):8-14.
54. Schwebke JR, Gaydos CA, Nyirjesy P, Paradis S, Kodsi S, and Cooper CK. Diagnostic Performance of a Molecular Test versus Clinician Assessment of Vaginitis. *J Clin Microbiol*. 2018;56(6).
55. Fredricks DN, Fiedler TL, Thomas KK, Oakley BB, and Marrazzo JM. Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *J Clin Microbiol*. 2007;45(10):3270-6.
56. Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Van Simaey L, De Ganck C, De Backer E, Temmerman M, and Vaneechoutte M. Comparison between Gram stain and culture for the characterization of vaginal microflora: definition of a distinct grade that resembles grade I microflora and revised categorization of grade I microflora. *BMC Microbiol*. 2005;5:61.
57. Verstraelen H and Verhelst R. Bacterial vaginosis: an update on diagnosis and treatment. *Expert Rev Anti Infect Ther*. 2009;7(9):1109-24.
58. Schwiertz A, Taras D, Rusch K, and Rusch V. Throwing the dice for the diagnosis of vaginal complaints? *Ann Clin Microbiol Antimicrob*. 2006;5:4.
59. Donders GG. Definition and classification of abnormal vaginal flora. *Best Pract Res Clin Obstet Gynaecol*. 2007;21(3):355-73.
60. Taylor-Robinson D, Morgan DJ, Sheehan M, Rosenstein IJ, and Lamont RF. Relation between Gram-stain and clinical criteria for diagnosing bacterial vaginosis with special reference to Gram grade II evaluation. *Int J STD AIDS*. 2003;14(1):6-10.
61. Lamont RF and Taylor-Robinson D. The role of bacterial vaginosis, aerobic vaginitis, abnormal vaginal flora and the risk of preterm birth. *BJOG*. 2010;117(1):119-20; author reply 120-1.
62. Shipitsyna E, Roos A, Datcu R, Hallen A, Fredlund H, Jensen JS, Engstrand L, and Unemo M. Composition of the vaginal microbiota in women of reproductive age--sensitive and specific molecular diagnosis of bacterial vaginosis is possible? *PLoS One*. 2013;8(4):e60670.
63. Rumyantseva T, Shipitsyna E, Guschin A, and Unemo M. Evaluation and subsequent optimizations

of the quantitative AmpliSens Florocenosis/Bacterial vaginosis-FRT multiplex real-time PCR assay for diagnosis of bacterial vaginosis. *APMIS*. 2016;124(12):1099-1108.

64. Hay PE, Morgan DJ, Ison CA, Bhide SA, Romney M, McKenzie P, Pearson J, Lamont RF, and Taylor-Robinson D. A longitudinal study of bacterial vaginosis during pregnancy. *Br J Obstet Gynaecol*. 1994;101(12):1048-53.
65. Ison CA and Hay PE. Validation of a simplified grading of Gram stained vaginal smears for use in genitourinary medicine clinics. *Sex Transm Infect*. 2002;78(6):413-5.
66. Chawla R, Bhalla P, Chadha S, Grover S, and Garg S. Comparison of Hay's criteria with Nugent's scoring system for diagnosis of bacterial vaginosis. *Biomed Res Int*. 2013;2013:365194.
67. Lamont RF, Hudson EA, Hay PE, Morgan DJ, Modi V, Ison CA, and Taylor-Robinson D. A comparison of the use of Papanicolaou-stained cervical cytological smears with Gram-stained vaginal smears for the diagnosis of bacterial vaginosis in early pregnancy. *Int J STD AIDS*. 1999;10(2):93-7.
68. Ling Z, Kong J, Liu F, Zhu H, Chen X, Wang Y, Li L, Nelson KE, Xia Y, and Xiang C. Molecular analysis of the diversity of vaginal microbiota associated with bacterial vaginosis. *BMC Genomics*. 2010;11:488.
69. Lamont RF, Sobel JD, Akins RA, Hassan SS, Chaiworapongsa T, Kusanovic JP, and Romero R. The vaginal microbiome: new information about genital tract flora using molecular based techniques. *BJOG*. 2011;118(5):533-549.
70. Srinivasan S, Hoffman NG, Morgan MT, Matsen FA, Fiedler TL, Hall RW, Ross FJ, McCoy CO, Bumgarner R, Marrazzo JM, and Fredricks DN. Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PLoS One*. 2012;7(6):e37818.
71. Dols JA, Molenaar D, van der Helm JJ, Caspers MP, de Kat Angelino-Bart A, Schuren FH, Speksnijder AG, Westerhoff HV, Richardus JH, Boon ME, Reid G, de Vries HJ, and Kort R. Molecular assessment of bacterial vaginosis by Lactobacillus abundance and species diversity. *BMC Infect Dis*. 2016;16:180.
72. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, and Forney LJ. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U S A*. 2011;108 Suppl 1:4680-7.
73. Fredricks DN, Fiedler TL, and Marrazzo JM. Molecular identification of bacteria associated with bacterial vaginosis. *N Engl J Med*. 2005;353(18):1899-911.
74. Wertz J, Isaacs-Cosgrove N, Holzman C, and Marsh TL. Temporal Shifts in Microbial Communities in Nonpregnant African-American Women with and without Bacterial Vaginosis. *Interdiscip Perspect Infect Dis*. 2008;2008:181253.
75. Thies FL, Konig W, and Konig B. Rapid characterization of the normal and disturbed vaginal microbiota by application of 16S rRNA gene terminal RFLP fingerprinting. *J Med Microbiol*. 2007;56(Pt 6):755-61.
76. Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Delanghe J, Van Simaey L, De Ganck C, Temmerman M, and Vaneechoutte M. Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between Atopobium vaginae, Gardnerella vaginalis and bacterial vaginosis. *BMC Microbiol*. 2004;4:16.
77. Antonio MA, Hawes SE, and Hillier SL. The identification of vaginal Lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species. *J Infect Dis*. 1999;180(6):1950-6.

78. Witkin SS, Mendes-Soares H, Linhares IM, Jayaram A, Ledger WJ, and Forney LJ. Influence of vaginal bacteria and D- and L-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: implications for protection against upper genital tract infections. *MBio*. 2013;4(4).
79. Vaneechoutte M. Lactobacillus iners, the unusual suspect. *Res Microbiol*. 2017;168(9-10):826-836.
80. Verstraelen H, Verhelst R, Claeys G, De Backer E, Temmerman M, and Vaneechoutte M. Longitudinal analysis of the vaginal microflora in pregnancy suggests that L. crispatus promotes the stability of the normal vaginal microflora and that L. gasseri and/or L. iners are more conducive to the occurrence of abnormal vaginal microflora. *BMC Microbiol*. 2009;9:116.
81. Petrova MI, Reid G, Vaneechoutte M, and Lebeer S. Lactobacillus iners: Friend or Foe? *Trends Microbiol*. 2017;25(3):182-191.
82. Donders GG, Vereecken A, Bosmans E, Dekeersmaecker A, Salembier G, and Spitz B. Definition of a type of abnormal vaginal flora that is distinct from bacterial vaginosis: aerobic vaginitis. *BJOG*. 2002;109(1):34-43.
83. Gray LA and Barnes ML. Vaginitis in Women, Diagnosis and Treatment. *Am J Obstet Gynecol*. 1965;92:125-36.
84. Paavonen J and Brunham RC. Bacterial Vaginosis and Desquamative Inflammatory Vaginitis. *N Engl J Med*. 2018;379(23):2246-2254.
85. Muzny CA, Taylor CM, Swords WE, Tamhane A, Chattopadhyay D, Cerca N, and Schwebke JR. An Updated Conceptual Model on the Pathogenesis of Bacterial Vaginosis. *J Infect Dis*. 2019;220(9):1399-1405.
86. van de Wijgert JH, Borgdorff H, Verhelst R, Crucitti T, Francis S, Verstraelen H, and Jespers V. The vaginal microbiota: what have we learned after a decade of molecular characterization? *PLoS One*. 2014;9(8):e105998.
87. Onderdonk AB, Delaney ML, and Fichorova RN. The Human Microbiome during Bacterial Vaginosis. *Clin Microbiol Rev*. 2016;29(2):223-38.
88. Freitas AC and Hill JE. Quantification, isolation and characterization of Bifidobacterium from the vaginal microbiomes of reproductive aged women. *Anaerobe*. 2017;47:145-156.
89. De Seta F, Campisciano G, Zanotta N, Ricci G, and Comar M. The Vaginal Community State Types Microbiome-Immune Network as Key Factor for Bacterial Vaginosis and Aerobic Vaginitis. *Front Microbiol*. 2019;10:2451.
90. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, and Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*. 2014;505(7484):559-63.
91. Sonnenburg ED and Sonnenburg JL. The ancestral and industrialized gut microbiota and implications for human health. *Nat Rev Microbiol*. 2019;17(6):383-390.
92. Lax S, Smith DP, Hampton-Marcell J, Owens SM, Handley KM, Scott NM, Gibbons SM, Larsen P, Shogan BD, Weiss S, Metcalf JL, Ursell LK, Vazquez-Baeza Y, Van Treuren W, Hasan NA, Gibson MK, Colwell R, Dantas G, Knight R, and Gilbert JA. Longitudinal analysis of microbial interaction between humans and the indoor environment. *Science*. 2014;345(6200):1048-52.
93. Oh J, Byrd AL, Deming C, Conlan S, Program NCS, Kong HH, and Segre JA. Biogeography and individuality shape function in the human skin metagenome. *Nature*. 2014;514(7520):59-64.
94. Koumans EH, Markowitz LE, Hogan V, and Group CBW. Indications for therapy and treatment

recommendations for bacterial vaginosis in nonpregnant and pregnant women: a synthesis of data. *Clin Infect Dis*. 2002;35(Suppl 2):S152-72.

95. Lamont RF, Morgan DJ, Wilden SD, and Taylor-Robinson D. Prevalence of bacterial vaginosis in women attending one of three general practices for routine cervical cytology. *Int J STD AIDS*. 2000;11(8):495-8.
96. Park HY, Kim CR, Huh IS, Jung MY, Seo EY, Park JH, Lee DY, and Yang JM. Staphylococcus aureus Colonization in Acute and Chronic Skin Lesions of Patients with Atopic Dermatitis. *Ann Dermatol*. 2013;25(4):410-6.
97. Kong HH, Oh J, Deming C, Conlan S, Grice EA, Beatson MA, Nomicos E, Polley EC, Komarow HD, Program NCS, Murray PR, Turner ML, and Segre JA. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res*. 2012;22(5):850-9.
98. Brunner PM, Guttman-Yassky E, and Leung DY. The immunology of atopic dermatitis and its reversibility with broad-spectrum and targeted therapies. *J Allergy Clin Immunol*. 2017;139(4S):S65-S76.
99. Kennedy K, Heimall J, and Spergel JM. Advances in atopic dermatitis in 2017. *J Allergy Clin Immunol*. 2018;142(6):1740-1747.
100. Eichenfield LF, Tom WL, Berger TG, Krol A, Paller AS, Schwarzenberger K, Bergman JN, Chamlin SL, Cohen DE, Cooper KD, Cordoro KM, Davis DM, Feldman SR, Hanifin JM, Margolis DJ, Silverman RA, Simpson EL, Williams HC, Elmets CA, Block J, Harrod CG, Smith Begolka W, and Sidbury R. Guidelines of care for the management of atopic dermatitis: section 2. Management and treatment of atopic dermatitis with topical therapies. *J Am Acad Dermatol*. 2014;71(1):116-32.
101. Niemeyer-van der Kolk T, van der Wall HEC, Balmforth C, Van Doorn MBA, and Rissmann R. A systematic literature review of the human skin microbiome as biomarker for dermatological drug development. *Br J Clin Pharmacol*. 2018;84(10):2178-2193.
102. Donders GGG, Bellen G, Grinceviciene S, Ruban K, and Vieira-Baptista P. Aerobic vaginitis: no longer a stranger. *Res Microbiol*. 2017.
103. Sobel JD. Vulvovaginal candidosis. *Lancet*. 2007;369(9577):1961-71.
104. Marrazzo JM, Thomas KK, Fiedler TL, Ringwood K, and Fredricks DN. Relationship of specific vaginal bacteria and bacterial vaginosis treatment failure in women who have sex with women. *Ann Intern Med*. 2008;149(1):20-8.
105. Deng ZL, Gottschick C, Bhuju S, Masur C, Abels C, and Wagner-Dobler I. Metatranscriptome Analysis of the Vaginal Microbiota Reveals Potential Mechanisms for Protection against Metronidazole in Bacterial Vaginosis. *mSphere*. 2018;3(3).
106. Kirkcaldy RD, Augostini P, Asbel LE, Bernstein KT, Kerani RP, Mettenbrink CJ, Pathela P, Schwebke JR, Secor WE, Workowski KA, Davis D, Braxton J, and Weinstock HS. Trichomonas vaginalis antimicrobial drug resistance in 6 US cities, STD Surveillance Network, 2009-2010. *Emerg Infect Dis*. 2012;18(6):939-43.
107. Schellenberg JJ, Paramel Jayaprakash T, Withana Gamage N, Patterson MH, Vaneechoutte M, and Hill JE. Gardnerella vaginalis Subgroups Defined by cpn60 Sequencing and Sialidase Activity in Isolates from Canada, Belgium and Kenya. *PLoS One*. 2016;11(1):e0146510.
108. Janulaitiene M, Paliulyte V, Grinceviciene S, Zakareviciene J, Vladisauskiene A, Marcinkute A, and Pleckaityte M. Prevalence and distribution of Gardnerella vaginalis subgroups in women with and without bacterial vaginosis. *BMC Infect Dis*. 2017;17(1):394.

109. Ahmed A, Earl J, Retchless A, Hillier SL, Rabe LK, Cherpes TL, Powell E, Janto B, Eutsey R, Hiller NL, Boissy R, Dahlgren ME, Hall BG, Costerton JW, Post JC, Hu FZ, and Ehrlich GD. Comparative genomic analyses of 17 clinical isolates of Gardnerella vaginalis provide evidence of multiple genetically isolated clades consistent with subspeciation into genovars. *J Bacteriol*. 2012;194(15):3922-37.
110. Vaneechoutte M, Guschin A, Van Simaey L, Gansemans Y, Van Nieuwerburgh F, and Cools P. Emended description of Gardnerella vaginalis and description of Gardnerella leopoldii sp. nov., Gardnerella piotii sp. nov. and Gardnerella swidsinskii sp. nov., with delineation of 13 genomic species within the genus Gardnerella. *Int J Syst Evol Microbiol*. 2019;69(3):679-687.
111. Muzny CA and Schwebke JR. Biofilms: An Underappreciated Mechanism of Treatment Failure and Recurrence in Vaginal Infections. *Clin Infect Dis*. 2015;61(4):601-6.
112. Castro J, Franca A, Bradwell KR, Serrano MG, Jefferson KK, and Cerca N. Comparative transcriptomic analysis of Gardnerella vaginalis biofilms vs. planktonic cultures using RNA-seq. *NPJ Biofilms Microbiomes*. 2017;3:3.
113. Verstraelen H and Swidsinski A. The biofilm in bacterial vaginosis: implications for epidemiology, diagnosis and treatment: 2018 update. *Curr Opin Infect Dis*. 2019;32(1):38-42.
114. Ferris MJ, Masztal A, Aldridge KE, Fortenberry JD, Fidel PL, Jr., and Martin DH. Association of Atopobium vaginae, a recently described metronidazole resistant anaerobe, with bacterial vaginosis. *BMC Infect Dis*. 2004;4:5.
115. Ferris MJ, Norori J, Zozaya-Hinchliffe M, and Martin DH. Cultivation-independent analysis of changes in bacterial vaginosis flora following metronidazole treatment. *J Clin Microbiol*. 2007;45(3):1016-8.
116. Xiao B, Wu C, Song W, Niu X, Qin N, Liu Z, and Xu Q. Association Analysis on Recurrence of Bacterial Vaginosis Revealed Microbes and Clinical Variables Important for Treatment Outcome. *Front Cell Infect Microbiol*. 2019;9:189.
117. Beeton ML and Spiller OB. Antibiotic resistance among Ureaplasma spp. isolates: cause for concern? *J Antimicrob Chemother*. 2017;72(2):330-337.
118. Austin MN, Beigi RH, Meyn LA, and Hillier SL. Microbiologic response to treatment of bacterial vaginosis with topical clindamycin or metronidazole. *J Clin Microbiol*. 2005;43(9):4492-7.
119. Schuyler JA, Mordechai E, Adelson ME, Sobel JD, Gygax SE, and Hilbert DW. Identification of intrinsically metronidazole-resistant clades of Gardnerella vaginalis. *Diagn Microbiol Infect Dis*. 2016;84(1):1-3.
120. Rosca AS, Castro J, Sousa LGV, and Cerca N. Gardnerella and vaginal health: the truth is out there. *FEMS Microbiol Rev*. 2019.
121. Seite S, Flores GE, Henley JB, Martin R, Zelenkova H, Aguilar L, and Fierer N. Microbiome of affected and unaffected skin of patients with atopic dermatitis before and after emollient treatment. *J Drugs Dermatol*. 2014;13(11):1365-72.
122. Gonzalez ME, Schaffer JV, Orlow SJ, Gao Z, Li H, Alekseyenko AV, and Blaser MJ. Cutaneous microbiome effects of fluticasone propionate cream and adjunctive bleach baths in childhood atopic dermatitis. *J Am Acad Dermatol*. 2016;75(3):481-493 e8.
123. Seite S, Zelenkova H, and Martin R. Clinical efficacy of emollients in atopic dermatitis patients - relationship with the skin microbiota modification. *Clin Cosmet Investig Dermatol*. 2017;10:25-33.
124. Kwon S, Choi JY, Shin JW, Huh CH, Park KC, Du MH, Yoon S, and Na JI. Changes in Lesional and Non-

lesional Skin Microbiome During Treatment of Atopic Dermatitis. *Acta Derm Venereol*. 2018.

125. Grice EA, Kong HH, Conlan S, Deming CB, Davis J, Young AC, Program NCS, Bouffard GG, Blakesley RW, Murray PR, Green ED, Turner ML, and Segre JA. Topographical and temporal diversity of the human skin microbiome. *Science*. 2009;324(5931):1190-2.
126. Oh J, Byrd AL, Park M, Program NCS, Kong HH, and Segre JA. Temporal Stability of the Human Skin Microbiome. *Cell*. 2016;165(4):854-66.
127. Flores GE, Caporaso JG, Henley JB, Rideout JR, Domogala D, Chase J, Leff JW, Vazquez-Baeza Y, Gonzalez A, Knight R, Dunn RR, and Fierer N. Temporal variability is a personalized feature of the human microbiome. *Genome Biol*. 2014;15(12):531.
128. Collaborators GBDCoD. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet*. 2018;392(10159):1736-1788.
129. Disease GBD, Injury I, and Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet*. 2017;390(10100):1211-1259.
130. Biesbroek G, Tsivtsivadze E, Sanders EA, Montijn R, Veenhoven RH, Keijser BJ, and Bogaert D. Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children. *Am J Respir Crit Care Med*. 2014;190(11):1283-92.
131. Bosch A, Levin E, van Houten MA, Hasrat R, Kalkman G, Biesbroek G, de Steenhuijsen Piters WAA, de Groot PCM, Pernet P, Keijser BJF, Sanders EAM, and Bogaert D. Development of Upper Respiratory Tract Microbiota in Infancy is Affected by Mode of Delivery. *EBioMedicine*. 2016;9:336-345.
132. Bosch A, de Steenhuijsen Piters WAA, van Houten MA, Chu M, Biesbroek G, Kool J, Pernet P, de Groot PCM, Eijkemans MJC, Keijser BJF, Sanders EAM, and Bogaert D. Maturation of the Infant Respiratory Microbiota, Environmental Drivers, and Health Consequences. A Prospective Cohort Study. *Am J Respir Crit Care Med*. 2017;196(12):1582-1590.
133. Man WH, van Houten MA, Merelle ME, Vlieger AM, Chu M, Jansen NJG, Sanders EAM, and Bogaert D. Bacterial and viral respiratory tract microbiota and host characteristics in children with lower respiratory tract infections: a matched case-control study. *Lancet Respir Med*. 2019;7(5):417-426.
134. Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N, Holt BJ, Hales BJ, Walker ML, Hollams E, Bochkov YA, Grindle K, Johnston SL, Gern JE, Sly PD, Holt PG, Holt KE, and Inouye M. The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe*. 2015;17(5):704-15.
135. Krone CL, van de Groep K, Trzcinski K, Sanders EA, and Bogaert D. Immunosenescence and pneumococcal disease: an imbalance in host-pathogen interactions. *Lancet Respir Med*. 2014;2(2):141-53.
136. Pettigrew MM, Laufer AS, Gent JF, Kong Y, Fennie KP, and Metlay JP. Upper respiratory tract microbial communities, acute otitis media pathogens, and antibiotic use in healthy and sick children. *Appl Environ Microbiol*. 2012;78(17):6262-70.
137. Bogaert D and van Belkum A. Antibiotic treatment and stewardship in the era of microbiota-oriented diagnostics. *Eur J Clin Microbiol Infect Dis*. 2018;37(5):795-798.
138. Bunker CB and Shim TN. Male genital lichen sclerosus. *Indian J Dermatol*. 2015;60(2):111-7.

139. Lewis FM, Tatnall FM, Velangi SS, Bunker CB, Kumar A, Brackenbury F, Mohd Mustapa MF, and Exton LS. British Association of Dermatologists guidelines for the management of lichen sclerosus, 2018. *Br J Dermatol*. 2018;178(4):839-853.
140. Bunker CB, Patel N, and Shim TN. Urinary voiding symptomatology (micro-incontinence) in male genital lichen sclerosus. *Acta Derm Venereol*. 2013;93(2):246-8.
141. Price LB, Liu CM, Johnson KE, Aziz M, Lau MK, Bowers J, Ravel J, Keim PS, Serwadda D, Wawer MJ, and Gray RH. The effects of circumcision on the penis microbiome. *PLoS One*. 2010;5(1):e8422.
142. Liu CM, Hungate BA, Tobian AA, Serwadda D, Ravel J, Lester R, Kigozi G, Aziz M, Galiwango RM, Nalugoda F, Contente-Cuomo TL, Wawer MJ, Keim P, Gray RH, and Price LB. Male circumcision significantly reduces prevalence and load of genital anaerobic bacteria. *MBio*. 2013;4(2):e00076.
143. Han YW. Fusobacterium nucleatum: a commensal-turned pathogen. *Curr Opin Microbiol*. 2015;23:141-7.
144. Sears CL and Garrett WS. Microbes, microbiota, and colon cancer. *Cell Host Microbe*. 2014;15(3):317-28.
145. Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, Barnes R, Watson P, Allen-Vercoe E, Moore RA, and Holt RA. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res*. 2012;22(2):299-306.
146. Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, Ojesina AI, Jung J, Bass AJ, Tabernero J, Baselga J, Liu C, Shivdasani RA, Ogino S, Birren BW, Huttenhower C, Garrett WS, and Meyerson M. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res*. 2012;22(2):292-8.
147. Al-Hebshi NN, Nasher AT, Maryoud MY, Homeida HE, Chen T, Idris AM, and Johnson NW. Inflammatory bacteriome featuring Fusobacterium nucleatum and Pseudomonas aeruginosa identified in association with oral squamous cell carcinoma. *Sci Rep*. 2017;7(1):1834.
148. Zhao H, Chu M, Huang Z, Yang X, Ran S, Hu B, Zhang C, and Liang J. Variations in oral microbiota associated with oral cancer. *Sci Rep*. 2017;7(1):11773.
149. Krisanaprakornkit S, Kimball JR, Weinberg A, Darveau RP, Bainbridge BW, and Dale BA. Inducible expression of human beta-defensin 2 by Fusobacterium nucleatum in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. *Infect Immun*. 2000;68(5):2907-15.
150. Yin L and Dale BA. Activation of protective responses in oral epithelial cells by Fusobacterium nucleatum and human beta-defensin-2. *J Med Microbiol*. 2007;56(Pt 7):976-87.
151. Gursoy UK, Kononen E, and Uitto VJ. Stimulation of epithelial cell matrix metalloproteinase (MMP-2, -9, -13) and interleukin-8 secretion by fusobacteria. *Oral Microbiol Immunol*. 2008;23(5):432-4.
152. Zhang G and Rudney JD. Streptococcus cristatus attenuates Fusobacterium nucleatum-induced cytokine expression by influencing pathways converging on nuclear factor-kappaB. *Mol Oral Microbiol*. 2011;26(2):150-63.
153. Park SR, Kim DJ, Han SH, Kang MJ, Lee JY, Jeong YJ, Lee SJ, Kim TH, Ahn SG, Yoon JH, and Park JH. Diverse Toll-like receptors mediate cytokine production by Fusobacterium nucleatum and Aggregatibacter actinomycetemcomitans in macrophages. *Infect Immun*. 2014;82(5):1914-20.
154. Uitto VJ, Baillie D, Wu Q, Gendron R, Grenier D, Putnins EE, Kanervo A, and Firth JD. Fusobacterium nucleatum increases collagenase 3 production and migration of epithelial cells. *Infect Immun*. 2005;73(2):1171-9.

155. Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, and Han YW. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/beta-catenin signaling via its FadA adhesin. *Cell Host Microbe*. 2013;14(2):195-206.
156. Binder Gallimidi A, Fischman S, Revach B, Bulvik R, Maliutina A, Rubinstein AM, Nussbaum G, and Elkin M. Periodontal pathogens Porphyromonas gingivalis and Fusobacterium nucleatum promote tumor progression in an oral-specific chemical carcinogenesis model. *Oncotarget*. 2015;6(26):22613-23.
157. Chen Y, Peng Y, Yu J, Chen T, Wu Y, Shi L, Li Q, Wu J, and Fu X. Invasive Fusobacterium nucleatum activates beta-catenin signaling in colorectal cancer via a TLR4/P-PAK1 cascade. *Oncotarget*. 2017;8(19):31802-31814.
158. Carroll IM, Ringel-Kulka T, Siddle JP, Klaenhammer TR, and Ringel Y. Characterization of the fecal microbiota using high-throughput sequencing reveals a stable microbial community during storage. *PLoS One*. 2012;7(10):e46953.
159. Dominianni C, Wu J, Hayes RB, and Ahn J. Comparison of methods for fecal microbiome biospecimen collection. *BMC Microbiol*. 2014;14:103.
160. Cardona S, Eck A, Cassellas M, Gallart M, Alastrue C, Dore J, Azpiroz F, Roca J, Guarner F, and Manichanh C. Storage conditions of intestinal microbiota matter in metagenomic analysis. *BMC Microbiol*. 2012;12:158.
161. Shaw AG, Sim K, Powell E, Cornwell E, Cramer T, McClure ZE, Li MS, and Kroll JS. Latitude in sample handling and storage for infant faecal microbiota studies: the elephant in the room? *Microbiome*. 2016;4(1):40.
162. Kennedy NA, Walker AW, Berry SH, Duncan SH, Farquarson FM, Louis P, Thomson JM, Consortium UIG, Satsangi J, Flint HJ, Parkhill J, Lees CW, and Hold GL. The impact of different DNA extraction kits and laboratories upon the assessment of human gut microbiota composition by 16S rRNA gene sequencing. *PLoS One*. 2014;9(2):e88982.
163. Wu GD, Lewis JD, Hoffmann C, Chen YY, Knight R, Bittinger K, Hwang J, Chen J, Berkowsky R, Nessel L, Li H, and Bushman FD. Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. *BMC Microbiol*. 2010;10:206.
164. Hendolin PH, Paulin L, and Ylikoski J. Clinically applicable multiplex PCR for four middle ear pathogens. *J Clin Microbiol*. 2000;38(1):125-32.
165. Vandeventer PE, Weigel KM, Salazar J, Erwin B, Irvine B, Doebler R, Nadim A, Cangelosi GA, and Niemz A. Mechanical disruption of lysis-resistant bacterial cells by use of a miniature, low-power, disposable device. *J Clin Microbiol*. 2011;49(7):2533-9.
166. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, and Glockner FO. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res*. 2013;41(1):e1.
167. Kopylova E, Navas-Molina JA, Mercier C, Xu ZZ, Mahe F, He Y, Zhou HW, Rognes T, Caporaso JG, and Knight R. Open-Source Sequence Clustering Methods Improve the State Of the Art. *mSystems*. 2016;1(1).
168. Westcott SL and Schloss PD. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. *PeerJ*. 2015;3:e1487.

169. Theis KR, Romero R, Winters AD, Greenberg JM, Gomez-Lopez N, Alhousseini A, Bieda J, Maymon E, Pacora P, Fettweis JM, Buck GA, Jefferson KK, Strauss JF, 3rd, Erez O, and Hassan SS. Does the human placenta delivered at term have a microbiota? Results of cultivation, quantitative real-time PCR, 16S rRNA gene sequencing, and metagenomics. *Am J Obstet Gynecol*. 2019;220(3):267 e1-267 e39.
170. Hornung BVH, Zwittink RD, Ducarmon QR, and Kuijper EJ. Response to: 'Circulating microbiome in blood of different circulatory compartments' by Schierwagen et al. *Gut*. 2019.
171. Pereira-Marques J, Hout A, Ferreira RM, Weber M, Pinto-Ribeiro I, van Doorn LJ, Knetsch CW, and Figueiredo C. Impact of host DNA and sequencing depth on the taxonomic resoluation of whole metagenome sequencing for microbiome analysis. *Front Microbiol*. 2019;10(1277).
172. Chrystoja CC and Diamandis EP. Whole genome sequencing as a diagnostic test: challenges and opportunities. *Clin Chem*. 2014;60(5):724-33.
173. Amrane SL, J. C. Metagenomic and clinical microbiology. *Human Microbiome Journal*. 2018;9:1-6.