The microbiome in primary Sjögren's syndrome

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CHAPTER 7

General discussion and future perspectives

GENERAL DISCUSSION

Homeostasis between the host immune system and the microbiome is crucial for the health status of the host. Disturbance of homeostasis between the human host and microbiome (designated as dysbiosis) is suggested as a major factor in the development of chronic inflammatory diseases (1–5). PSS is characterized by local and systemic chronic inflammation, but the role of the human microbiome has only scarcely been studied thus far. At the time we reviewed the literature (**chapter 2**), no human studies on the microbiome in pSS patients were available. However, based on the results of experimental studies and clinical studies performed in patients with other systemic autoimmune diseases (i.e., rheumatoid arthritis and systemic lupus erythematosus), we proposed a mechanism by which dysbiosis of the gut microbiome may lead to systemic inflammation in pSS. After publication of this review, three reports on the oral microbiome in pSS patients were published. All three studies included relatively low numbers of pSS patients and controls (≤10 individuals per group) (5–7). The main shortcoming of these studies was, however, that they did not answer the question whether changes in the oral microbiome of pSS patients are specific for the disease. Fecal samples from pSS patients were assessed in two earlier studies, of which only one used full 16S rRNA gene sequencing (5). The results from this study suggested that pSS patients have a specific gut microbiome in comparison to healthy subjects. However, the conclusions that could be drawn from the results of this study by de Paiva et al. were limited by the small sample size (10 pSS patients) and the possible effect of confounding through technical bias (5). The second study investigating fecal samples from pSS patients used a 16S rRNA gene based dysbiosis test (8). Severe dysbiosis of the gut microbiome was observed more often in pSS patients than in controls, but the true bacterial composition was not analyzed (8).

The overall aim of this thesis was to find disease-specific associations between pSS and the microbiota composition in three specific body sites, viz. the oral cavity, gut and vagina. In this chapter, we discuss our key-findings on the relation between pSS and the microbiota composition at the three aforementioned locations.

Connection between the oral microbiome and pSS

To assess whether pSS patients have a specific oral microbiome, we performed 16S rRNA sequencing on oral washings from 36 pSS patients, 85 non-SS sicca patients (i.e., disease controls) and 14 healthy controls (HCs) to capture to overall bacterial composition in the mouth (**chapter 3**). Both pSS patients and non-SS sicca patients (i.e., patients with sicca complaints not fulfilling the 2016 ACR/EULAR classification criteria for pSS (9)) had a lower salivary flow rate compared to normal values. The bacterial composition of the oral microbiome in pSS and non-SS patients differed significantly from that of HCs, but

was largely similar between pSS and non-SS sicca patients. Individual pSS patients could not be identified based on the microbiome in oral washings, due to a large heterogeneity in oral bacterial composition between pSS-individuals. An important finding was that stimulated salivary flow rate explained more of the variation in bacterial composition between individual samples (i.e., samples from pSS, non-SS sicca and HCs) than disease-status (9% versus 5%, respectively). Furthermore, salivary flow rate significantly correlated with the relative abundance of individual bacterial genera (viz., *Haemophilus*, *Neisseria* and *Lactobacillus*). From the results of this study, we conclude that the level of salivary flow has a stronger influence on the overall oral microbiome (as assessed in oral washings) than the underlying disease.

Oral washing samples provide a more general overview of the (largely planktonic) oral microbiome, but do not capture the oral mucosa microbiome specifically. Therefore, in **chapter 4**, we describe a study in which we performed 16S rRNA sequencing on buccal swab samples from the same group of 37 pSS-patients and 86 non-SS sicca patients as in chapter 3. The microbiota composition in buccal swab samples was compared with buccal swabs from 24 HCs and 103 population controls. We showed that also the buccal mucosa microbiome of pSS and non-SS sicca patients differs significantly from that of HCs, but is largely similar between pSS and non-SS patients. Disease-status (pSS, non-SS sicca or HCs) and salivary flow rate contributed almost equally to the variation in bacterial composition between individuals (3.8% and 4.3%, respectively). Twelve of the 19 pSS-associated taxa (compared to HCs) significantly correlated with salivary flow rate, confirming that changes in the oral microbiome of pSS patients are largely related to reduced salivary flow.

The results from the studies described in **chapter 3** and **4**, suggest that the changed oral microbiome in pSS patients is more likely to be a symptom of underlying disease, rather than an etiological factor for pSS. When stimulated whole salivary flow rate was taken into account, *Granulicatella* and *Bergeyella* lost their association with pSS compared with non-SS sicca, whereas lower *Streptococcus* remained associated with pSS. This indicated that *Streptococcus* relative abundance is associated with pSS, independent of salivary flow rate. However, non-SS sicca patients also showed a lower *Streptococcus* relative abundance in oral washings compared with healthy controls, suggesting that similar factors, other than salivary flow rate, in pSS and non-SS sicca patients result in lower *Streptococcus* relative abundance.

All patients in the studies described in **chapter 3** and **4** (i.e., pSS and non-SS sicca), were referred for a diagnostic work-up for pSS. Patients with oral dryness were assigned either to the pSS or non-SS sicca group, based on the ACR/EULAR classification criteria for pSS (9). It is likely that early-pSS patients (i.e., oral dryness patients not yet fulfilling the ACR/

EULAR criteria for pSS) were present in the non-SS sicca group. The presence of early-pSS patients in the non-SS sicca group may have contributed to a comparable oral microbiota composition in pSS as in non-SS sicca patients. Currently there are no criteria to define early-pSS, and therefore the presence of early-pSS patients could not be assessed.

Factors contributing to the oral microbiota composition

Based on the outcomes found in **chapter 3** and **4**, we thus established the salivary flow rate to be an important factor in determining the overall oral microbiota composition and relative abundance of individual bacterial taxa (**chapter 3** and **4**). However, how exactly salivary flow rate influences the oral microbiota composition remains largely unknown. Salivary flow rate explained 4% of the microbiota composition in buccal swabs and 9% in oral washings, which leaves a large part of the oral microbiota composition to be explained by other factors. Furthermore, the correlations between salivary flow and relative abundance of specific taxa were moderately strong at best (Spearman's *rho*≤0.55). These results indicate that other factors than salivary flow rate also contribute to the composition of oral microbiota.

Recently, five host-related biochemical salivary parameters (i.e., pH and buffered pH, chitinase activity, lysozyme activity, albumin and mucin 5B concentration) were reported to associate with specific oral microbiome clusters in saliva samples from healthy young adults (10). Currently, the most well-described effect of salivary parameters on oral microbiota is the relation between pH and the abundance of *Lactobacillus* species (see section 1.1.2 below). The salivary parameters assessed in the study by Zaura et al. were not assessed in the studies described in **chapters 3-5** (10). Therefore, it remains currently unknown whether the changes in oral microbiota composition in patients with reduced salivary secretion are mainly caused by dryness (or low oral humidity), a change in concentration of salivary mucins, proteins or peptides, or – most likely – a combination of these factors.

In **chapter 5**, we identified two more factors that explained a substantial proportion of the overall oral microbiota composition. Self-reported number of own teeth (i.e., edentulous, 1-9, 10-19 or >20 teeth) explained 15% and the self-reported condition of gums (i.e., very bad, bad, moderate, good, very good or excellent) explained 16% of the overall oral microbiota composition in pSS and SLE patients. This indicates that the overall oral microbiota composition is directly influenced by the number of teeth of an individual and his/her health of gums. Possibly, loss of teeth (and hence loss of dental surface and periodontal area in the mouth), results in a lower relative abundance of dental biofilm-associated bacteria. Therefore, in all three studies assessing the oral microbiome (chapters 3-5), we took dental status into account as possible confounding factor in the assessment of associations between pSS and oral microbiota.

Together, the results from our studies indicate that future studies assessing the relationship between the oral microbiome and systemic and oral diseases, should take into account salivary flow rate in the statistical analyses. Especially because salivary flow rate is affected, and mostly reduced, in a large variety of diseases, as well as that many frequently prescribed medications may result in changed salivary gland functioning (11,12). Furthermore, salivary biochemical parameters (including pH, mucins and antimicrobial peptides) and oral health status (dental and periodontal) of a study participant should also be included in future clinical studies assessing the oral microbiome.

Effect of salivary flow rate on individual bacterial taxa

In **chapters 3** and **4**, we showed that salivary flow rate correlated significantly with the relative abundance of three bacterial genera (i.e., *Haemophilus*, *Neisseria* and *Lactobacillus*) in buccal swab and oral washing samples. Previous studies using culture techniques have shown that patients with hyposalivation have higher *Lactobacillus* counts in oral washing and dental plaque samples (13–15). In **chapters 3** and **4**, we further elucidated the connection between *Lactobacillus* and salivary flow. We showed that the relative abundance of *Lactobacillus* in buccal mucosa and oral washing samples correlates with the unstimulated and stimulated whole salivary flow rate of an individual. *Lactobacillus* species are gram-positive, rod-shaped, facultative anaerobic bacteria and are well-known for their role in caries progression (16,17). Lactobacilli are aciduric (i.e., they can survive in a low pH environment) and acidogenic (i.e., they produce lactate-acid as byproduct of anaerobic fermentation of carbohydrates) (16). Reduced salivary flow rate results in a lower pH in saliva and dental plaque, because the homeostatic functions of saliva are reduced or lost (14,18,19). Saliva has three homeostatic functions to maintain a normal pH in the oral cavity: buffer-capacity, dilution of sugars and the clearance of both sugars and bacteria (20). Loss of clearance capacity increases the time that carbohydrates remain in the oral cavity. Carbohydrates are the main source for acidogenic bacteria, thereby increasing the lactic-acid production. Due to loss of buffer capacity and dilution capacity in patients with hyposalivation, the acids produced by bacteria cannot be counteracted to increase the oral pH and hence, the proportion of aciduric bacteria increases at the cost of bacteria that grow less well in an acidic environment. Thus, reduced salivary flow in pSS patients results in the loss of pH homeostasis in the oral cavity, thereby promoting the growth of aciduric bacteria, such as *Lactobacillus* species (20).

Through the use of 16S rRNA gene sequencing we were able to study the relation between many different bacterial taxa and the salivary flow rate of an individual. In both buccal swabs and oral washings, we showed that higher salivary flow rates resulted in higher relative abundances of genera *Haemophilus* and *Neisseria*. Both *Haemophilus* and *Neisseria* are considered to be part of the 'core oral microbiome' in healthy individuals (21,22). In individuals

with a stimulated salivary flow rate below 1 mL/min, the relative abundance of *Haemophilus* and *Neisseria* was even undetectable with sequence depths of 8000 reads/sample in oral washings and 1000 reads/sample in buccal swabs. Thus, higher relative abundance of *Haemophilus* and *Neisseria* may be considered characteristic of a healthy oral environment, maintained by adequate salivary flow. We speculate here that *Haemophilus* and *Neisseria* species are more sensitive to a dry environment and therefore their relative abundances decrease under circumstances of lower water availability. Indeed, the species *Haemophilus paraphrophilus* grows better under circumstances of increased moisture content *in vitro* (23). Also, the relative abundance of *Haemophilus* species on the conjunctiva appears to be higher in months with higher air humidity than in dry months in Madrid (24). The effect of humidity on *Neisseria* species is less well described, although it has been reported that invasive meningococcal disease, caused by *Neisseria meningitidis*, occurs more often when the absolute humidity in the air is at its lowest (25). However, whether this is related to a higher relative abundance of *Neisseria meningitidis* or host-related factors is unknown. To further test the effect of differences in humidity on the growth of *Haemophilus* and *Neisseria* species, bacterial species in these genera can be cultured using a relative humidity gradient technique, as described by de Goffau et al. (26).

Restoration of a healthy microbiome in pSS patients

Restoration of a healthy oral microbiome in pSS patients seems to require restoration of the unique properties of saliva. Several studies have assessed the effect of saliva substitutes on the oral microbiota, but these mainly showed antibacterial effects, which are not necessarily beneficial for the host microbiota composition (27,28). A recent study showed that a probiotic fermented milk drink has no significant effect on the salivary microbiota composition in healthy individuals (29). Specific bacteria from the probiotic milk drink persisted to a certain extent in the salivary microbiome, but the change lasted less than 24 hours (29). These results indicate that the environment of the oral cavity is relatively resistant to colonization with newly introduced bacteria under constant environmental conditions. In pSS patients and other patients with hyposalivation, a similar result can be expected when trying to restore dysbiosis to a healthy oral microbiome. A healthy oral microbiome in pSS patients can probably only be restored when the oral environment is restored to a healthy state. Thus, the main challenge in restoring a healthy oral microbiome in patients with hyposalivation, is to design a treatment with a prolonged effect on the oral microbiota that compensates for the loss of those functions of saliva that are needed for maintenance of a healthy oral microbiome. Therefore, studies that aim to find new ways to restore salivary gland function in pSS patients will also contribute to restoring a healthy oral microbiome. Pringle et al. recently showed that salivary gland stem cells in pSS patients are lower in number and less able to differentiate compared with salivary gland stem cells

from healthy controls (30). Therefore, a possible treatment option could be a transfer of newly generated autologous salivary gland stem cells to the salivary glands of pSS patients in order to regenerate the salivary gland parenchyma and to restore salivary gland function (30,31). However, this possible new treatment method is in an early phase of investigation and has not yet been tested on animals or patients.

Connection between the gut microbiome and pSS

To take a first step in investigating the possible role of gut microbiota in the etiopathogenesis of pSS, we collected fecal samples from 39 pSS and 30 SLE patients (included as disease-controls) and compared this with 965 fecal samples from individuals from the general population (i.e., LifeLines DEEP participants) (32,33). 16S rRNA gene sequencing was performed on fecal samples as well as on buccal swab and oral washing samples from the same pSS and SLE patients. The results of this study, described in **chapter 5**, show that the fecal microbiota composition from pSS and SLE patients differs significantly from that of population controls, but is largely similar between pSS and SLE patients. The gut microbiome of pSS and SLE patients was characterized by lower bacterial richness, lower Firmicutes/Bacteroidetes ratio and higher relative abundance of *Bacteroides* species compared with the gut microbiome of population controls. In the total patient population (i.e., pSS and SLE patients together, n=69), we showed that the relative abundance of *Actinomyces* and *Lactobacillus* in the oral cavity correlated significantly with their relative abundance in the gut, suggesting that the oral microbiota composition influences the gut microbiota composition. Thus, the results of this study indicate that pSS and SLE patients share a largely similar gut microbiota composition that distinguishes patients from general population controls.

The shared altered gut microbiota composition in pSS and SLE patients may suggest that the gut microbiome plays a common role in the etiology of both diseases. However, gut microbiota changes may also be the effect of changes in the intestinal environment as a consequence to similarities in genetic risk factors, pathogenesis (specifically B cell hyperactivity), or disturbances in tolerance induction and maintenance (34–38). Secretory immunoglobulin A (SIgA) in the gut lumen is one of the most important host factors that regulate the composition of the gut microbiota (39). Possibly, the immunopathogenic mechanisms underlying pSS and SLE lead to an altered SIgA repertoire in the gut lumen, subsequently causing an altered gut microbiome compared with healthy controls. A technique called IgA-sequencing (IgA-SEQ), introduced by Palm et al. (40), could be used to assess which bacteria in fecal samples are IgA-bound or –unbound in pSS and SLE patients. Using IgA-SEQ on fecal samples may give more insight in the cause and effect relationship between the gut microbiome and pSS and SLE.

1.2.1 Low richness characterizes the gut microbiome of pSS and SLE patients

We observed a lower bacterial richness, but similar diversity in fecal samples from pSS and SLE patients compared with those from population controls. The number of observed bacterial taxa in a sample (i.e., richness) depends on the number of sequenced reads (41). In our study, the number of reads did not differ between pSS patients, SLE patients and population controls (Wilcoxon, $p>0.1$). Furthermore, all fecal samples were rarefied to an equal sequencing depth (i.e., 8000 reads/sample) in order to minimize possible technical differences between samples. Therefore, we can conclude that the lower richness in pSS and SLE patients is most likely a true biological association, rather than a technical effect.

Recently, a study from a Chinese research group compared the gut microbiota composition from 45 SLE patients with that of 48 healthy controls and also found a similar lower richness, and no difference in diversity of the gut microbiome in SLE patients (42). Richness of the gut microbiome has been reported to inversely correlate with ocular and systemic disease severity in a relatively small study in 10 pSS patients (5). As discussed in **chapter 5**, low bacterial richness can be a sign of systemic inflammation, characterized by increased C reactive protein (CRP) concentration in plasma and higher number of lymphocytes in blood (41). Possibly, lower bacterial richness is related to a higher systemic inflammatory state in pSS patients, reflected by a higher disease severity. In our study, we did not include disease activity index scores of pSS and SLE patients, because the European Sjögren's syndrome Disease Activity Index (ESSDAI) and systemic lupus erythematosus disease activity index (SLEDAI) were not assessed during routine clinical visits (43,44). Therefore, we were unable to assess the connection between richness of the gut microbiome and disease activity in pSS and SLE patients. A possible way to assess the connection between the gut microbiome, systemic inflammation and disease severity in pSS and SLE patients would be to perform a longitudinal study in which fecal samples are collected at multiple time points, together with serum biochemistry and disease activity scoring.

The role of Bacteroides species in pSS

We observed a markedly higher relative abundance of genus *Bacteroides* in the gut microbiome of pSS and SLE patients compared with the gut microbiome of population controls. *Bacteroides* species are one of the most abundant commensal bacteria in the gut microbiome. Two recent studies reported that cross-reactivity between *Bacteroides* species and anti-Ro60 autoantibodies may be a mechanism underlying pSS and SLE pathogenesis (45,46). Szymula et al. performed a Basic Local Alignment Search Tool (BLAST) analysis to find mimicry peptides in microbiota resembling epitopes of the Ro60 protein (45). They showed that a peptide from the von Willebrand factor type A domain protein, present in three *Bacteroides* species (viz., *B. finegoldii*, *B. instetinalis* and *B. fragilis*), was able to activate

Ro60-reactive T cell hybridoma cells (45). More recently, Greiling et al. showed that lysates of *Bacteroides thetaiotaomicron* can bind to serum from anti-Ro60-positive SLE patients (46). Moreover, B and T cell responses to the Ro60-protein occurred after monocolonization of mice with *B. thetaiotaomicron* and lead to enhanced lupus-like disease (46).

In our study, the relative abundance of *B. thetaiotaomicron* was higher in both pSS and SLE patients than in population controls, but only statistically significant for SLE patients. We found no association between *B. thetaiotaomicron* relative abundance and anti-Ro60 autoantibody presence in the serum of pSS and SLE patients. In addition to the higher relative abundance of *B. thetaiotaomicron*, we observed a significantly higher abundance of three other *Bacteroides* species, *B. vulgatus*, *B. uniformis* and *B. ovatus* in pSS and SLE patients. Therefore, the role of *B. thetaiotaomicron* and other *Bacteroides* species should further be studied in clinical and experimental studies.

As a consequence of 16S rRNA gene sequencing, our analyses relied on measures of relative abundance of bacteria, which might under- or overestimate the biological meaning of associations between bacteria and pSS. Currently, it is recommended that microbiome research should include both relative and quantitative profiling, in order to more precisely define the relation between bacteria and the host (47). The combination of next-generation sequencing and quantitative microbiome profiling will especially be useful in assessing the role of *Bacteroides* species in pSS and its connection with the Ro60 autoantigen. Furthermore, now that we and others have identified *Bacteroides* species as possible players in pSS and SLE, conventional culturing techniques can be used to test cross-reactivity between *Bacteroides* species and patient serum with Ro60 autoantibodies (46). Another possible way to study the role of *Bacteroides* species in the etiology of pSS is via *Bacteroides* colonization of SS-prone mice and subsequent assessment of symptom onset and anti-Ro60 autoantibody production (46,48,49).

Treatment of pSS through dietary intervention

A high relative abundance of *Bacteroides* species is characteristic for the gut microbiome of pSS and SLE patients. Dietary interventions for pSS and SLE could therefore focus on reducing *Bacteroides* species, although it is unknown whether such interventions will reduce symptoms. The relative abundance of *Bacteroides* species increases during an animal-based diet, but was not shown to significantly decrease during a plant-based diet in healthy individuals (50). Recently, a study showed that a whole grain diet reduced systemic low-grade inflammation (i.e., serum C-reactive protein, IL-6 and IL-1β) compared to a refined-grain diet in individuals at risk for metabolic disease, although the gut microbiome did not change (51).

Interestingly, none of the pSS and SLE patients included in the gut microbiome study (**chapter 5**) were vegetarian (i.e., did not consume meat in any meal) according to the Dutch Healthy Diet food frequency questionnaire (52). Although a plant-based diet did not reduce *Bacteroides* species in healthy individuals (53), a vegetarian diet may be proposed as dietary advice in pSS and SLE patients in which the relative abundance of *Bacteroides* is high. Two studies from before the 16S sequencing era, assessed vegan and vegetarian diet interventions in RA patients and showed that these diets had a positive effect on disease symptoms (54,55). Thus, conducting a randomized controlled trial in pSS patients in which patients are allocated to continue their usual diet or start a vegetarian diet, is a possible method to assess whether diet can reduce pSS-symptoms, and whether this is dependent on changes in the gut microbiome. In the future, individual gut microbiome profiling should form the basis of personalized dietary, pre- or probiotic interventions.

The oral – gut microbiome connection

In **chapter 5** we showed that the relative abundance of specific bacterial genera in the oral microbiome – i.e., *Actinomyces* and *Lactobacillus* – correlated significantly with their relative abundance in the gut microbiome. Furthermore, the statistically significant difference in *Actinomyces* relative abundance between pSS patients and SLE patients in the oral microbiome was also observed in the gut microbiome. These results suggest that the relative abundance of specific microbiota in the oral microbiome is still present in the gut microbiome. Although this may seem reasonable, it is quite remarkable that the relative abundance of a specific genus seems to remain relatively stable throughout the gastrointestinal tract, considering, for example, the low pH in the stomach. Increasing the pH in the stomach, through the use of proton pump inhibitors (PPIs), may result in a less hostile gastric environment for oral bacteria to survive. This is supported by the recent finding that individuals using PPIs have a gut microbiome that is more similar to the oral microbiome, compared with the gut microbiome of non-PPI users (56). Using the same population controls (i.e., LifeLines participants) as in the study by Imhann et al. (56), we also observed a significantly higher relative abundance of 'oral genera' *Streptococcus*, *Lactobacillus* and *Actinomyces* in fecal samples from patients and population controls using PPIs (n=99) compared with non-PPI users (n=935) in the total study population of 1034 individuals.

In a recent study, Atarashi et al. showed that ectopic colonization of oral *Klebsiella* species into the gut of mice induced an inflammatory response in the gut mucosa (57). Thus, under certain circumstances, oral bacteria can be intestinal pathobionts, exacerbating inflammation of the gut mucosa (57). However, the connection between the oral and gut

microbiome is only recently being explored as a potential relevant source of gut dysbiosis in human disease, and not many studies have analyzed both oral and fecal samples from the same individual (3,58).

To further elucidate the role of the oral microbiome in shaping the gut microbiome, strain-specific bacterial identification is needed to precisely track bacterial species from mouth to feces. Furthermore, the role of saliva and salivary components (i.e., salivary flow, mucins, antimicrobial peptides and SIgA) in the oral-gut microbiome connection has not yet been explored in humans. In cows, it was recently shown that the composition of salivary microbiota coated with SIgA, more closely represented the rumen (i.e., cow stomach) microbiota composition than the total saliva microbiota composition (i.e., SIgA-coated and SIgA-uncoated microbiota) (59). This result suggests that salivary SIgA impacts the microbiome from the oral cavity to the stomach, and possibly further along the gastrointestinal tract. Indeed, a correlation has been observed between salivary SIgA and *Bifidobacterium* and *Lactobacillus* species in the fecal microbiome of infants up to 12 months old (60). The authors concluded that the diversity in *Bifidobacterium* species may enhance the maturation of the mucosal SIgA system (60). However the reversed possibility that higher salivary SIgA results in higher *Bifidobacterium* and *Lactobacillus* species in the gut may also happen.

In pSS patients, the salivary SIgA concentration is not significantly different from controls (61), but the low salivary flow in pSS patients may reduce the absolute availability of SIgA in the oral cavity. Thus, the role of salivary SIgA on the gut microbiota composition in healthy individuals and pSS patients remains largely unknown so far. Since SIgA is a major factor in maintaining homeostasis between intestinal mucosal immunity and the microbiota, it is well worth investigating the role of salivary SIgA on the gut microbiome in pSS patients. Performing IgA-SEQ on oral and fecal samples can potentially reveal how coating of bacteria with salivary SIgA influences the total IgA-coated gut microbiome (40).

Vaginal microbiome in women with pSS

We hypothesized that vaginal dryness in pSS women may also be associated with alterations in the vaginal microbiome, similar to how oral dryness affects the oral microbiome in pSS patients. In the study described in **chapter 6**, we compared the vaginal microbiome of 9 premenopausal women with pSS and vaginal dryness complaints with that of 8 premenopausal control women without vaginal dryness complaints. 16S rRNA sequencing was performed on cervicovaginal lavages and endocervical swabs. Much to our surprise, the results of this study suggested that pSS-associated vaginal dryness in premenopausal women does not negatively influence the homeostasis of the vaginal microbiome. We speculate that this is due to the unique vaginal microbiota composition, which is strongly

dominated by acid-producing lactobacilli. Thus, pSS-associated dryness of epithelial surfaces, has a larger effect on the oral than on the vaginal microbiota composition. Consequently, whereas changes in the oral microbiome may lead to increased prevalence of caries and oral candidiasis in pSS patients, the normal vaginal microbiome in pSS patients probably protects women with pSS from vaginal infections in a similar way as in healthy women. To our knowledge, no studies are available that report on the prevalence of vaginal infections in women with pSS.

Most likely, the difference between a changed oral versus unchanged vaginal microbiota composition in pSS patients, is explained by a changing versus stable local environment. In the oral cavity, low salivary flow results in lower salivary pH (18,62), whereas in the vagina, we did not observe a change of pH in women with pSS compared with controls. Moreover, it is currently unknown if subjective vaginal dryness correlates with objective measurements of vaginal dryness in women with pSS.

Lactobacillus species are considered the most important microbiota in a healthy vaginal microbiome (63). In the oral cavity, we (**chapters 3** and **4**) and others (10,64) rarely observed lactobacilli in oral samples from healthy individuals. This illustrates that the same bacterial genera are associated with both homeostasis and dysbiosis, depending on where they colonize the host.

Future perspectives

Knowledge on the role of the host-microbiome relationship in human health and disease has greatly increased over the past decade. The studies described in this thesis show new insights of how pSS is connected to the oral, gut and vaginal microbiome. Together, these studies provide a basis for future studies assessing the connection between pSS and the human microbiome. Moreover, our results are also relevant for patients with oral dryness not caused by pSS (**chapters 3** and **4**) and for SLE patients (**chapter 5**).

Since we now partially elucidated the connection between pSS and the oral microbiome, future studies should focus more on unraveling the pathogenesis of the two most frequently observed oral complications in pSS patients: cervical caries and oral candidiasis. Good treatment options exist for dental caries and oral candidiasis, but these treatments come at the cost of loss of dental material (in caries), financial burden and possible side effects of medication (for caries and oral candidiasis). Therefore, prevention of caries and oral candidiasis would greatly benefit pSS patients and other patients with low salivary flow and increased risks of these oral complications.

Integration of modern research techniques, such as whole genome sequencing (WGS), proteomics, metabolomics and fluorescence in situ hybridization techniques (as performed by Zijnge et al. (17) and Mark Welch et al. (65)), can be used to get a more complete picture of how microbial biofilms are organized on teeth and oral mucosa, and what the role of saliva is in these processes. If the microbial processes, leading to caries and oral candidiasis in pSS patients, are better understood, new preventive strategies may be developed. For example, specific salivary proteins/peptides, bacterial peptides, pre- or probiotics, may be incorporated in food, an oral gel or a toothpaste, to target a specific microbial interaction in biofilm formation, thereby preventing growth of dental plaque biofilm or outgrowth of Candida.

Several studies have shown that probiotic treatment with different *Lactobacillus* species may be effective in the prevention of Candida overgrowth (66–69). Interestingly, despite lactobacilli being present in higher amounts in the oral cavity of pSS patients and non-SS patients with hyposalivation, oral candidiasis also occurs more often in patients with hyposalivation than in healthy individuals. Possibly, lactobacilli prevent Candida overgrowth to some extent, but in patients with oral candidiasis, the protective mechanism of lactobacilli fails to stop further Candida overgrowth. Probiotic treatment with *Lactobacillus* species may therefore still be an optional preventive treatment for oral candidiasis in pSS patients. The more acidic oral environment in pSS patients may even better facilitate oral colonization with probiotic lactobacilli than in healthy individuals. However, a stable and healthy oral microbiome in pSS patients will probably require daily intake of probiotics (29), as long as the patients' own salivary secretion is not restored.

We describe in **chapter 5** the largest study to date on the gut microbiome in pSS patients and we are the first to use SLE patients as disease controls for pSS in microbiome studies. To confirm our findings, more studies are needed, which include larger numbers of pSS and SLE patients. WGS and quantitative microbiome profiling should be used to more precisely define the gut microbiota composition than can be done with 16S rRNA gene sequencing. To strengthen the findings of a microbiome-pSS connection, a study should be performed simultaneously in different regions of the world (e.g., Europe, North America and South East Asia), in order to account for ethnicity and geographical factors.

Moving forward from association to causality is crucial to elucidate the role of gut microbiota in pSS. In human studies, causality can be studied in longitudinal studies where fecal samples are collected before and after disease onset. Studies using longitudinal fecal sample collections suggest that gut microbiota are involved in the onset of type 1 diabetes (70–72). However, this is much more difficult for pSS than for type 1 diabetes, because type 1 diabetes generally starts early in life, whereas pSS usually develops around the age of

50. Moreover, genetic susceptibility for pSS is less evident than for type 1 diabetes, which makes selection of individuals susceptible for pSS difficult. Individuals susceptible for pSS might also be identified based on oral and ocular dryness symptoms. Early-pSS patients can be defined as patients with signs and symptoms suggestive of pSS, but not fulfilling the classification criteria, and who are clinically suspected to develop pSS in the future. However, there is no internationally accepted definition for early-pSS and the description of early-pSS patients above will result in a very heterogeneous group of 'early-patients'. Despite these limitations, we showed that inclusion of 'symptom-controls' (i.e., non-SS sicca patients) provided more insight in the cause-effect relationship between the oral microbiome and pSS (**chapter 3** and **4**). Thus, to further assess the cause-effect relationship between the gut microbiome and pSS, fecal samples from patients with dry mouth and/or eye symptoms, suspect for pSS, should be collected prospectively. In the University Medical Center Groningen (UMCG), the Netherlands, an estimated 25% of these patients is presumed to develop pSS-characteristic features over the years (i.e., anti-Ro/SSA, anti-La/SSB autoantibodies and/or focal sialadenitis), which will classify them as having pSS (9). Of note, a subject that not fulfil the classification criteria for pSS can still be clinically diagnosed as pSS. If specific changes in gut microbiota precede the onset of pSS, and the gut microbiome stays unchanged in non-SS sicca patients, then a causal effect of the gut microbiome in pSS etiopathogenesis becomes more plausible.

Experimental mouse studies have also shown to be a valuable method of studying the role of gut microbiota in pSS and SLE (46,49,73–75). An experimental mouse study that has not yet been performed for pSS, is the inoculation of mice with fecal material from pSS patients and subsequent assessment of the development of pSS-features in these mice. Based on the results described in chapter 5, pSS-prone mice may also be monocolonized with different *Bacteroides* species to assess the effect of these species on disease progression and severity.

Finally, pSS patients who are treated with abatacept, as participant in the currently ongoing Abatacept Sjögren Active Patient (ASAP) III trial in the UMCG, can provide more insight in how adaptive immunity affects the gut microbiome. Specifically, the role of antigen presentation to T cells in regulating the gut microbiome can be studied in pSS patients receiving abatacept, because abatacept blocks the co-stimulating signal between antigen presenting cells (APCs) and T cells. Therefore, fecal and oral samples are currently collected from pSS patients before and during abatacept therapy, as part of the ASAP III study in the UMCG.

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