Microbial community interactions: effects of probiotics on oral microcosms
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Chapter VI - Summary, general discussion, future research
This thesis is a part of the Dutch Technology Foundation (STW) project entitled “Probing dynamic microbial community interactions”. The ultimate goal of our research was to gain a general insight in the dynamic interplay between environmental (and host induced) factors and the composition, activity and physiology of microbial communities, and to understand how this interplay is responsible for maintenance of health and, more specifically, initiation and progression of disease. A very crucial prerequisite for microbial community studies is to have access to a high throughput technique for microbial identification and quantification. Relying on isolation and identification of multiple microbial species by phenotypic tests, that are typically time-consuming and laborious, will limit these studies to only few species, usually ‘main pathogens’. Within this project we therefore aimed to develop and exploit a cost efficient, easy to handle and accurate technique that allows high throughput screening of the composition and dynamics of oral microbial communities.

As a particular application, but also a specific aim of this thesis, we undertook to understand the effects of a perturbation by two specific probiotic strains: Lactobacillus salivarius W24 (Chapter II) and Lactobacillus rhamnosus GG (LGG) (Chapter IV, V) on the composition, acidogenicity and cariogenic potential of an oral microbial community. We employed saliva-derived microcosms as a model for such a complex community (Chapter II, III, IV). A targeted method, named multiplex-dependent ligation probe amplification (MLPA), that allows high throughput screening of the community composition was developed during this project. Chapters III, IV and V address applicability and validation of this method for community profiling.

In Chapter II we assessed the perturbation of saliva-derived microcosms by the probiotic strain L. salivarius W24. This strain is included in commercial products aimed to enhance (recovery of) intestinal health. For community profiling we used a denaturing gradient gel electrophoresis (DGGE) fingerprinting method. Experimental results showed that W24 was not able to form a biofilm on its own or to enter an existing biofilm. However, W24 was able to establish into a saliva-derived microbial community if W24 and saliva were inoculated simultaneously.
From this we conclude that to form a biofilm, W24 requires not only a surface for attachment, but also an interaction with other microorganisms or their products. With regard to the clinical situation, this could mean that W24 may establish on dental surfaces within newly developing dental biofilm if it is provided to the oral cavity right after tooth brushing.

W24 belongs to the homofermentative lactic acid bacteria that are known to be aciduric and acidogenic. Once W24 has established in the microbial communities, it affected the ecology of the communities grown in the presence of sucrose: it reduced the pH and decreased the microbial diversity of the microcosms. Our results suggest that this specific probiotic strain may therefore increase the cariogenic potential of the oral microbial community if it establishes in the oral cavity. With this study we demonstrated that a relatively simple test of probiotics interacting with the complex microbial community could be applicable in the screening of probiotic strains for their potential harmful effects in the oral cavity.

In Chapter III we describe the development and applicability of multiplex ligation-dependent probe amplification (MLPA) for the relative quantification of bacterial species in oral communities. MLPA is a new method, developed by researchers from Amsterdam, at MRC-Holland, in collaboration with the Free University of Amsterdam, for detection of various genetic disorders (Schouten et al. 2002).

A panel of MLPA probes to specifically identify a well-documented and representative set of oral microorganisms was designed at the genus and species-level. A majority of the MLPA probes were designed on the 16S rDNA gene – a standard for phylogenetic classification. For species that had too similar 16S rDNA sequences, the designed probe was based on a different gene. The MLPA probes were validated on i) DNA obtained from pure bacterial cultures, ii) on saliva samples from different donors, iii) on microcosm biofilms grown in a constant depth film fermentor (CDFF) and iv) on defined consortia biofilms.

MLPA was able to characterize the expected differences in composition of the microbial community. Moreover, when we compared the community profiles of consortium biofilm samples generated by DGGE (an open-end, untargeted method)
with MLPA (a targeted method), we found that the sensitivity of MLPA was considerably higher than that of DGGE. Therefore we conclude that the targeted approach (e.g., by MLPA) is the preferred method to characterize defined consortia.

A serious limitation for all DNA-probe based targeting methods is their reliance on publicly available full genome and 16S rDNA databases. The lower the coverage of a particular microorganism of interest is in the databases, the more likely that the designed probe will not be entirely specific for this organism. Future expansions of the sequence databases should improve the probe designing process.

The most elaborate part of the MLPA methodology was the designing and one by one validating of probes and probe-mixes. Developing of MLPA probe sets depending on the purpose of the study, with a limited number of microorganism-specific probes per kit, would be more feasible than a large, all microbial groups covering probe panel. Thus specific kits could be compiled for the study of caries, periodontitis or halitosis. Once the necessary probes are designed and validated, the MLPA method is fast, highly sensitive (able to discriminate between sequences that differ in only one nucleotide), simple to perform and inexpensive.

In chapter IV we assessed the interaction of the probiotic L. rhamnosus GG (LGG) strain with the cariogenic bacterium Streptococcus mutans in dual species biofilms and followed the effects of LGG on the cariogenic potential and microbial composition of saliva-derived microcosms.

Similarly to the probiotic strain W24 (Chapter II), LGG was able to establish into the saliva-derived microcosms if inoculated simultaneously with saliva. LGG inhibited the growth of mutans streptococci both in dual species biofilms and in the microcosms. Our findings are in line with clinical observations where mutans streptococci counts were decreased due to a regular exposure to LGG-containing products, indicating specific inhibition of mutans streptococci by this probiotic strain.

LGG did not have a significant effect on the cariogenic potential of the microcosms – it did not affect the pH lowering potential, the lactic acid production or the demineralization of dentin. From this we conclude that LGG is, at least, a non-
cariogenic probiotic strain. Our model, however, mimicked an extremely cariogenic situation: a rapid pH decrease due to sucrose fermentation, followed by a long period at low pH (pH 5) until the next medium refreshment. We chose such severe conditions to be able to model a pronounced shift in microbial ecology, which in turn was necessary to allow validation of the community profiling by MLPA. In the clinical situation low pH episodes are intermingled with a pH rise due to salivary buffering and clearance. We cannot rule out that in a less extreme situation LGG may have showed anti-cariogenic effects besides the already observed inhibition of mutans streptococci. To elucidate this, further experiments using clinically more relevant growth conditions, such as pH cycling (intermingled feast and famine periods of nutrients) and increased clearance of metabolic products (more frequent refreshment of the growth medium) should be performed.

On the validation part of the MLPA, we showed that this method was able to characterize the changes in the composition of the microcosms resulting from a change in the growth medium, the differences among the individual salivas and due to the perturbation with the foreign bacterium, LGG. The MLPA probe of S. mutans worked well in the panel of nine MLPA probes (Chapter III). However, when the panel was expanded to 20 probes as in this study (Chapter IV), the sensitivity of this probe in the MLPA reaction decreased markedly. For further applicability of MLPA on oral microbiota, a more sensitive S. mutans probe, possibly based on another gene rather than the 16S gene sequence, is required.

For this study (Chapter IV) we selected six saliva donors that were screened for low and high mutans streptococci (MS) counts in saliva. However, all microcosms, irrespective of the type of the inoculum, produced similar outcomes – low pH and severe demineralization of dentin. This again, could be due to the severity of the model used. By using a set of 20 selected microbial targets (MLPA probes) we could only partly describe the composition of the microcosms. It remained undisclosed which microorganisms other than mutans streptococci contributed to the cariogenicity of the model. This illustrates the limitations of an assessment method only based on selected species.
To answer this question we sequenced the hypervariable regions V5-V7 of the 16S rDNA of the inocula and microcosm samples obtained in Chapter IV by means of 454 pyrosequencing (Chapter V). Additionally, we compared the MLPA results (Chapter IV) with the output of this open-ended high-throughput approach of community profiling.

The microbiome profiles obtained from the six inocula were clearly dichotomized into two groups that corresponded to the pre-screening of the saliva into either low or high MS category. As could be expected when using a relatively simple in vitro model and growth medium, the microcosm microbiomes differed significantly from the inocula. Therefore even more surprising was our finding that individual microbiomes retained their high or low MS-character, even under severe cariogenic conditions, i.e., growth in the presence of sucrose. From this we conclude that the choice of the inoculum based on predefined criteria allows differential modeling of the ecological shifts in the saliva-derived microcosms. This certainly should be explored further with more clinically relevant models and different groups of inoculum donors, e.g., individuals with low or high caries activity, periodontal disease, halitosis etc.

The microbiome profiles of the microcosms showed that the presence of sucrose promoted the growth of veillonellae, which dominated both low and high MS Suc+ microcosms. Since all microcosms demineralized dentin severely, irrespective of the inoculum type (Chapter IV), we propose that acetate produced by veillonellae may have contributed to the demineralization of dentin. Additional experiments should be performed to further explore the role of veillonellae in the (dentin) caries process.

We compared the output of the two profiling methods – MLPA and 454 pyrosequencing. The results were comparable in discriminating the effects of inocula and the growth conditions. This confirms that MLPA is a valid method in the characterization of the microcosms. Furthermore, the data obtained by pyrosequencing will allow selection of relevant probe sequences for further investigation by the targeted approach.

Current 454 pyrosequencing technology is limited to 400 nt (nucleotide) short sequences, which together with the incomplete reference database prevents final
taxonomical assignment of the sequences to the species level. Future developments (longer read length) and a custom reference database of genomes of clinically relevant (oral) microorganisms will increase the applicability of 454 pyrosequencing in characterization of complex oral microbial communities.

**FUTURE RESEARCH ON PROBIOTICS AND ORAL HEALTH**

Probiotics have been extensively studied for their intestinal health promoting effects. Oral health effects have been either neglected or limited to assessment of inhibition of certain microorganisms, *e.g.*, mutans streptococci, yeasts. Due to basic differences between the intestinal system and the oral cavity, *e.g.*, presence of hard non-shedding surfaces in the oral cavity, one cannot simply translate the results from intestinal health studies to the oral field.

Clinical trials with orally administered probiotics still lack conclusive results on the beneficial effects of probiotics on oral health. For instance, the only two clinical trials on children exposed to probiotic-containing products *(NASE* et al. 2001; *STECKSEN-BLICKS* et al. 2009) showed no strong evidence of the effects of probiotics on dental caries – there was no statistical difference between group with and without probiotic consumption. Hence, more clinical trials with proper control groups should be performed to assess the effects of probiotics on oral health, especially on dental caries. The placebo must, in terms of overall composition, be comparable with products containing probiotics. The output parameters should not only focus on the main pathogens – *S. mutans*, lactobacilli but should address the microbial community as a whole.

Probiotics are widely used in commercial products. With regard to the oral health field, we have little knowledge on how probiotics interact with the oral microbial community *in vivo*. Why are the effects of probiotics highly dependent on a specific strain? Is that due to the properties of this probiotic strain or due to the commensal species of the host? What mechanisms lay behind the interactions between the probiotic strains and the host microbiome? Does the host, *e.g.*, its immune system in addition to its microbiome, dictate the impact of probiotics? These and more
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133 questions should be addressed in the future to get further insight into the influence of probiotics on health.

FUTURE RESEARCH ON MICROBIAL COMMUNITY INTERACTIONS

For decades, single pathogen approaches have governed the treatment and prevention directions, both in health care and in industry. For example, in the case of caries, current diagnostic tools and treatments are based on monitoring and reducing mutans streptococci counts in plaque and saliva. Although potent enough in reducing mutans streptococci, these treatments (e.g., chlorhexidine applications) were never shown to result in successful caries inhibition. Recent developments strongly suggest that dental plaque microorganisms act as a community rather than as separate individual species. Therefore, microbial community-profiling methods, e.g., targeted probe-based MLPA, developed during this project, or open-ended high throughput pyrosequencing used to validate the MLPA technique, are indispensible in oral health related studies. There are many possible applications for microbial community profiling tools, such as following the effects of treatments and individual risk assessment for oral diseases. In the near future, microbial activity rather than just microbial presence will need to be assessed, e.g., by probes based on mRNA gene sequences, or by mRNA-sequencing of complete microbiota (metatranscriptomes). These developments will bring additional challenges of data processing and interpretation, but should eventually narrow the gap between our current state of knowledge and our ultimate goal: gaining insights in dynamic microbial community interactions. Such insights are instrumental in obtaining a fundamental understanding and tackling the problems that these communities cause.