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Chapter I - Introduction

This chapter starts with an introduction on microbial communities and community interactions. Next, a brief overview on probiotics, especially two probiotic strains used (*Lactobacillus salivarius* W24 and *Lactobacillus rhamnosus* GG) will be given. The objective of the thesis will be described, followed by an outline of the thesis.

MICROBIAL COMMUNITIES

Basic properties of dental plaque biofilms

More than 300 years ago, Antonie van Leeuwenhoek (1632 – 1723) discovered a new research direction – oral microbiology. He observed the 'material' on teeth under his microscope and described this 'as a few living animalcules' (KURAMITSU *et al.* 2007). That 'material' nowadays is known as dental plaque biofilm (MARSH 2004) and the 'few living animalcules' are microorganisms that constitute the dental plaque biofilm.

Dental plaque biofilm is one of natural biofilms that contain communities of matrix-enclosed microorganisms attached to a surface (MARSH and MARTIN 1999). The oral cavity is continuously bathed with saliva, which keeps the conditions warm (35 – 36°C) and moist, at a pH between 6.5 and 7.3. This condition is optimal for the growth of many bacteria (MARSH 2003a). Recent analysis of more than 36,000 16S rDNA gene clones revealed approximately 1,200 predominant species in the oral cavity, of which 24% were named, 8% were cultivated but unnamed, and 68% were uncultivated species (DEWHIRST *et al.* 2010). A study utilizing 454 pyrosequencing revealed 3,621 and 6,888 species-level phylotypes present in saliva and plaque samples, respectively (KEIJSER *et al.* 2008). Furthermore, this study estimated overall diversity of human oral microbiota at approximately 26,000 phylotypes.

In saliva, the bacteria are either suspended or specifically congregated with other bacteria. If the bacteria are not attached to any surface, they will be swallowed. Due to the properties of the habitat, bacteria adhere, establish and become dominant at that distinct surface (MARSH 2006). Once attached, bacteria change their properties in order to adapt to the new habitat, *e.g.*, by altering the patterns of their gene expression (JEFFERSON 2004). This in turn causes severe problems in oral health –

dental caries, periodontitis, *etc*. A summary of properties of biofilms is given in Table 1.

Table 1. Summary of general properties of a biofilm

Open architecture – presence of channels and voids
Protection from host defenses and predators – colonization resistance
Protection from desiccation
Protection from antimicrobial agents
• Surface-associated phenotype
• Slow growth rate
• Poor penetration
• Inactivation/neutralization of inhibitors
Novel gene expression and phenotype
Persistence in flowing systems
Spatial and environmental heterogeneity
Spatial organization facilitating metabolic interactions
Elevated concentration of nutrients
Cell-to-cell communication

Adapted from Marsh & Martin, 1999 and Marsh *et al.*, 2011

Community interactions

Communities are defined as multi-species assemblages, in which organisms live together in a contiguous environment and interact with each other (KONOPKA 2009). Bacteria must interact cooperatively and competitively with other species to be able to reside in the microbial community (TEN CATE 2006). This results in stability of the microbial community at a site with regards to attachment, growth, metabolic communication, genetic exchange, quorum-sensing and survival of bacteria (MARSH 2005).

Cooperative interactions among oral bacteria enhance metabolic communication. For example, the interaction between *Streptococcus* and *Veillonella* species depends on lactic acid production and fermentation (HOJO *et al.* 2009). Streptococci produce and release lactic acid to the environment and veillonellae consume this lactic acid for

their survival. Veillonellae produce vitamin K, which supports the growth of *Prevotella* and *Porphyromonas* species (HOJO *et al.* 2009). In a quorum sensing process, some bacteria produce and release chemical signal molecules, *e.g.,* autoinducer 2 (AI-2) or a competence stimulating peptide (CSP) to the habitat, while the same or other bacteria detect the accumulation of these molecules and subsequently alter their gene expression and behaviour in response (CVITKOVITCH *et al.* 2003; SHAO and DEMUTH 2010).

Due to the limited space and nutrients, competitive interactions among oral bacteria occur frequently. Inhibitory substances – bacteriocins, hydrogen peroxide, *etc.* – are released to hamper the competitors. For instance, streptococci and enterococci produce a wide range of bacteriocins against other bacteria (NES *et al.* 2007), while *Streptococcus gordonii* and *Streptococcus sanguinis* generate hydrogen peroxide (H_2O_2) that inhibits the growth of *Streptococcus mutans* (KRETH *et al.* 2005; KRETH *et al.* 2008).

Living in a bacterial community provides potential benefits to the members of this community (MARSH 2005). These include:

a. A broader range of growth habitat

One critical factor for the formation and development of the microbial community is cell-to-cell interaction (KOLENBRANDER *et al.* 2005; KOLENBRANDER *et al.* 2010). As soon as teeth are cleaned, the enamel surfaces will become covered by a conditioning film (pellicle), which can facilitate early colonizing bacteria to adhere. The planktonic bacteria that cannot directly colonize may bind via receptors to the cell surface of the early colonizers.

Those bacteria that are not coaggregation partners interact cooperatively via coaggregation bridges. The interaction between *Prevotella loescheii*, *Streptococcus oralis* and *Actinomyces israelii* is an example of such a three way cooperation (KOLENBRANDER *et al.* 2002). *P. loescheii* acts as a bridge for coaggregation between *S. oralis* and *A. israelii*, spatially building up the microbial community.

b. Increased metabolic diversity and efficiency

Complex salivary molecules, *e.g.,* mucin-type glycoproteins comprise many types of carbohydrates. These carbohydrates are present in different

combinations. An individual bacterium will not be able to break down these complex molecules on its own. The molecules will be degraded efficiently only by several bacterial enzymes from a consortium of microorganisms (BRADSHAW *et al.* 1994).

c. *An enhanced resistance to environmental stress, antimicrobial agents and host defense systems*

Bacteria in biofilms are resilient to antimicrobial agents, and this effect is enhanced in microbial communities (MARSH 2005). For example, a lower susceptibility to chlorhexidine was found in defined oral species consortia compared to single species biofilms (KARA *et al.* 2006; PRATTEN *et al.* 1998a; WILSON *et al.* 1998). In addition, biofilms increase the opportunity for gene transfer among bacteria, leading to the spread of antimicrobial resistance in the community (MAH and O'TOOLE 2001).

d. *An enhanced ability to cause diseases*

Microbial communities display the properties that are more than just the sum of its components. Abscesses are examples of polymicrobial infections whereby organisms that individually cannot cause disease are able to do so when they are present as a consortium (MARSH 2005).

It has been shown that bacteria express different properties within dual- or multispecies biofilms (DENG *et al.* 2009; KARA *et al.* 2006; PERIASAMY *et al.* 2009). For example, the presence of *S. mutans* promotes growth of *Enterococcus faecalis* in biofilms (DENG *et al.* 2009). The cooperation of *S. oralis* and *Actinomyces naeslundii* promotes the formation of *Fusobacterium nucleatum* biofilms (PERIASAMY *et al.* 2009). Furthermore, differences between single-species biofilm – *S. mutans* or *Veillonella parvula* and dual-species biofilm - *S. mutans* and *V. parvula* with regard to response to chlorhexidine were observed (KARA *et al.* 2006). Dual-species biofilms were more resistant to chlorhexidine than single-species biofilms (KARA *et al.* 2006). However, by artificially choosing defined bacteria to study, the oral ecology cannot be investigated in its entire breadth. Thus, by shifting research from individual strains (the presumed main pathogens) and defined microbial consortia to higher complex

communities – dental plaque microcosms – we will be able to enhance our knowledge about oral ecology, especially the microbial interactions within the communities. Once we understand the complex interactions in such systems, we might be able to prevent the harmful effects the community (or biofilm) causes.

The use of a microcosm

Microcosm has been defined as 'a laboratory subset of the natural system from which it originates but from which it also evolves' (WIMPENNY 1988). According to Wimpenny, there are three main reasons for taking environmental biofilm samples and bringing them into the laboratory (WIMPENNY 1997):

1. It is more convenient to carry out experiments in a well-equipped laboratory than working in the field.
2. The environmental conditions for growing the microcosms can be better controlled than those in natural dental plaque.
3. The results of experiments are likely to be more reproducible than they might be in the field.

Microcosms derived from saliva (dental plaque microcosms) have been used broadly (MCBAIN *et al.* 2003; MCBAIN *et al.* 2005; PRATTEN and WILSON 1999; SISSONS *et al.* 2007; WILSON *et al.* 1998). Their use overcomes many of the problems with *ex vivo* plaque, such as: heterogeneity, reproducibility, the small quantities available, limited access, and also ethical issues (WONG and SISSONS 2001). Generally they are described as realistic, useful tools for a comprehensive study of the microbial ecology and physiology of the oral cavity (MCBAIN *et al.* 2005).

Biofilm models

An 'ideal' *in vitro* model should allow the study of plaque ecology, pathology and properties (SISSONS 1997). The model must be realistic, reflecting the plaque properties under investigation, and predictable when subjected to perturbation (SISSONS 1997). Commonly used models include simple microtiter plates (DJORDJEVIC *et al.* 2002; FILOCHE *et al.* 2007b; KUNZE *et al.* 2010; PITTS *et al.* 2003; STEPANOVIC *et al.*

2000) or high throughput Active Attachment Biofilm (AAB) models (DENG *et al.* 2009; EXTERKATE *et al.* 2010), and complex models such as the constant depth film fermentor (CDFF) (KINNIMENT *et al.* 1996a; KINNIMENT *et al.* 1996b; WILSON 1999) and the artificial mouth (FILOCHE *et al.* 2007a; SHU *et al.* 2000; SISSONS *et al.* 2007; SISSONS *et al.* 1991). The choice of a suitable model will depend on the purpose of the study. Throughout our project we have used the 24-wells microtiter plate, the AAB and the CDFF biofilm models. Therefore in short, the characteristics, advantages and disadvantages of each model will be discussed below:

- The microtiter plate model consists of 24 or 96 polyester plastic wells per plate. The biofilm forms on the plastic bottom/wall of the well or on the substratum – glass or hydroxylapatite disc inside the well.
- A novel active attachment biofilm model consists of a custom-made stainless steel lid with nylon clamps that can accommodate 24 substrata and fits into a 24-well plate. The substrata – glass, hydroxylapatite, or dentin/enamel discs – are positioned vertically and fitted into the wells without touching the wall of the well.
- A constant depth film fermentor (CDFF) consists of a glass vessel (18 cm diameter, 15 cm depth) with a stainless-steel turntable, ports for incoming medium, gas and sampling on the top plate and a port for spent medium (waste) in the bottom plate. The stainless-steel turntable (15 cm diameter) contains 15 polytetrafluoroethylene (PTFE) sample pans (2 cm diameter). Each sample pan carries 5 cylindrical holes containing PTFE plugs (5 mm in diameter), where a range of substrata can be placed on the top of the PTFE plugs. The turntable rotates under PTFE scraper blades that spread the incoming medium. By recessing the PTFE plugs to a predefined depth the thickness of the biofilm can be controlled.

Each model has its own advantages and disadvantages:

- The first two mentioned models are easy to handle in the laboratory. A wide variety of substrata can be used and various types of treatments can be compared. However, the biofilms cannot be grown for a long period. These models are used for high throughput screening.

- The CDFF model is a system that generates steady state biofilms. The environmental conditions used are mimicking the natural oral conditions such as nutrients available, surfaces for colonization, and a gas phase. Moreover, a reproducible biofilm with a constant thickness can be generated. The parameters – medium, flow rate, gas, *etc.* – can easily be controlled. Finally, it is useful to study the properties of mature biofilms, the effects of substrates and antimicrobial agents (PRATTEN *et al.* 1998b; PRATTEN and WILSON 1999; WILSON 1999). However, the growth rate of cells is not defined. Replicate biofilm samples are not grown independently within a CDFF chamber. For statistical analyses, an experiment should have independent outcomes. This makes CDFF experiments time-consuming and elaborative.

Artificial saliva – microcosm growth medium

Human saliva is produced by three pairs of major salivary glands (parotid, submandibular and sublingual) plus numerous minor salivary glands (DODDS *et al.* 2005). Saliva contains histatins, proline-rich proteins (PRPs), mucins, cystatins, statherin and enzymes: lysozyme, α-amylase, and albumin, *etc*. In addition, saliva is composed of a variety of electrolytes, including sodium, potassium, calcium, magnesium, bicarbonate, phosphate, and nitrogen sources such as urea and ammonia (HUMPHREY and WILLIAMSON 2001). For the growth of dental plaque microcosms in the laboratory, artificial saliva that mimics human natural saliva is used (MCBAIN *et al.* 2005; PRATTEN *et al.* 1998b; WONG and SISSONS 2001). One property of an artificial saliva medium is to support the growth of diverse microorganisms as it occurs in the human mouth. This project used the artificial saliva developed and described by McBain (MCBAIN *et al.* 2005). It includes heamin, vitamin K_1 (or menadione), required to support growth of *Porphyromonas* and *Prevotella* species (WONG and SISSONS 2001), mucin, peptone, tryptone, yeast extract, salts (sodium chloride, potassium chloride, calcium chloride) and cysteine hydrochloride – required to reduce the oxygen potential and to provide suitable anaerobic conditions.

Molecular techniques for the microbial community analyses

Isolation and identification of multiple microbial species by phenotypic tests, *e.g.*, cultivation, or the use of traditional cloning and sequencing methods (PASTER *et al.* 2006) are time-consuming and laborious methods. Recently several molecular techniques that are applicable in population studies have been developed. Each of these methods has its advantages and disadvantages. We have applied denaturing gradient gel electrophoresis (DGGE) (MUYZER *et al.* 1993), multiplex ligation-dependent probe amplification (MLPA) (SCHOUTEN *et al.* 2002) and the next generation 454 pyrosequencing technique (VOELKERDING *et al.* 2009). In the following, we give a brief description, advantages and disadvantages of the different techniques.

Description of the methods:

- Denaturing gradient gel electrophoresis (DGGE) is a method for determining the genetic diversity of complex microbial populations (MUYZER *et al.* 1993). The procedure is based on electrophoresis of PCR-amplified 16S rDNA fragments in a polyacrylamide gel containing a linearly increasing gradient of denaturants. In DGGE, DNA fragments of the same length but different base-pair sequence can be separated. This separation is based on the melting behavior of double-stranded DNA. The melting behavior depends on the base-pair composition of the DNA where a GC pair is stronger than an AT pair due to three instead of two hydrogen bonds between the nucleotides; and on the gradient of the denaturants, such as formamide and urea.
- Multiplex ligation-dependent probe amplification (MLPA) was originally developed for quantification of up to 50 different human DNA sequences in one reaction (OS and SCHOUTEN 2011; SCHOUTEN *et al.* 2002). Each MLPA probe consists of two oligonucleotides and should hybridize to adjacent target sequences. Only when these probes hybridize to immediately adjacent sections of the DNA strand the two probe oligonucleotides will be ligated. The ligation products will then be amplified in a single PCR reaction. In contrast to a standard multiplex PCR, a single pair of PCR primers is used for amplification of all MLPA reaction products. The resulting amount of amplification products

reflects the amount of the original target sequences. The amplified products are separated and quantified by capillary gel electrophoresis.

- Next generation sequencing (454 pyrosequencing) is a method for large scale parallel short read sequencing (VOELKERDING *et al.* 2009). Template DNA is fragmented, end-repaired and ligated to adapter oligonucleotides. The template is then diluted to single-molecule concentration, denatured, and hybridized to individual beads containing sequences complementary to adapter oligonucleotides. The beads are compartmentalized into water-in-oil microvesicles and clonally amplified by emulsion PCR. After amplification, the beads are deposited into individual picotiter-plate wells with sequencing enzymes. Loaded into the sequencer, the picotiter plate functions as a flow cell wherein iterative pyrosequencing is performed by successive flow addition of the 4 deoxynucleotide triphosphates (dNTPs). A nucleotide-incorporation event in a well containing clonally amplified template results in pyrophosphate (PPi) release with well-localized luminescence, which is transmitted through the fiber-optic plate and recorded on a charge-coupled device (CCD) camera. With the flow of each dNTP reagent, wells are imaged, analyzed for their signal-to-noise ratio, filtered according to quality criteria, and subsequently algorithmically translated into a linear sequence output.

The advantages and disadvantages of the three methods:

- MLPA is inexpensive, and can be performed by unspecialized molecular laboratories equipped with a sequencer. However, it is a targeted method where only a limited number (maximum 50) preselected known species can be detected and requires intensive probe preparation and validation step (OS and SCHOUTEN 2011; SCHOUTEN *et al.* 2002).
- DGGE is an open-ended method unbiased by known target species. The universal primers are used to anneal to any (or at least to the most) bacterial DNA present in the sample. Because of the short 16S rDNA fragment used, DGGE fragments originating from different organisms may have identical melting behavior and therefore cannot be separated in DGGE (ERCOLINI 2004). Another disadvantage of the DGGE method is the high detection limit – only

the abundant taxa (above the 1% of the total DNA) will be visualized on the gel (MUYZER and SMALLA 1998).

- 454 pyrosequencing is an open-ended method, which due to its high sequencing depth compared to other methods (*e.g.*, DGGE or cloning and sequencing) allows detection of rare phylotypes. However, short read length (currently about 400 nt) and an incomplete reference databases preclude full taxonomical assignment of the sequences obtained.

PROBIOTICS

Antibiotics have been widely used and misused in the past century (BARBOSA and LEVY 2000). Apart from side-effects to the host, some pathogens have become resistant to a range of antibiotics (TEUGHELS *et al.* 2008). A relationship between antibiotic use and the development of resistance has been demonstrated (BARBOSA and LEVY 2000). These developments have motivated researchers to focus on an alternative antimicrobial approach – 'probiotics' or a 'replacement therapy' (HILLMAN 2002). The use of this alternative microbial approach, in theory, is applicable to control any bacterial infection on a host surface (HILLMAN and SOCRANSKY 1987). In this approach, a natural species (probiotics) or laboratory derived strain (replacement therapy) is used to colonize the host surface where the pathogens reside (HILLMAN 2002). Once established, that strain would prevent the colonization or outgrowth of the pathogen by blocking the attachment sites, competing for essential nutrients or by other mechanisms through the synergetic and antagonistic interactions with members of the microbial communities (HILLMAN 2002). Ideally, the persistent colonization of that strain would result in a lifelong protection of the host (HILLMAN 2002; HILLMAN and SOCRANSKY 1987).

The term 'probiotic' is derived from the two Greek words: 'pro' and 'biotikos', meaning 'for life', in contrast to the term of 'antibiotic' – 'against life'. Probiotics were first described in 1965 by Lilly and Stillwell as 'substances produced by microorganisms which promote the growth of other microorganisms' (LILLY and STILLWELL 1965). Ever since, many definitions have been proposed to better describe the mechanisms of action and also the interactions with the host. The currently used

definition of probiotics is as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO 2006).

The proposed mechanisms of action of probiotics in the oral cavity (for details see reviews (DEVINE 2009; NG *et al.* 2009)) include:

a. Prevention of adhesion of pathogens

It has been shown that adhesion of pathogens to the host surfaces is reduced in the presence of probiotics. The probiotics either mask the surfaces or compete with the pathogens to attach to the surface resulting in the loss of adhesion of pathogens (HAUKIOJA *et al.* 2008a; VAN HOOGMOED *et al.* 2008; WEI *et al.* 2002).

b. Stimulation and modulation of the immune system

The adhesion of probiotics to the oral tissues promotes the health effect to the host (STAMATOVA and MEURMAN 2009). The epithelial layer in oral tissues functions as a physical barrier that is involved in immune response system (ISOLAURI *et al.* 2001). It has been shown that probiotics stimulate local immunity and modulate the inflammatory response (HACINI-RACHINEL *et al.* 2009). Although probiotic bacteria may influence the immune responses, the total immunoglobulin A (IgA) levels in saliva seem not to be affected by them (PAINEAU *et al.* 2008).

c. Killing or inhibition of growth of pathogens through release of antimicrobial substances, production of acid and hydrogen peroxide

Probiotics produce antimicrobial substance with potent inhibitory activity against a wide range of other bacteria. The inhibitory activity occurred at low pH (MEURMAN *et al.* 1995; SILVA *et al.* 1987) and was heat stable (SILVA *et al.* 1987). The releases of antimicrobial substances from lactobacilli strains facilitate the inhibition of anaerobic bacteria: *Clostridium*, Bacteroides, and *Bifibacterium* species; a member of the family *Enterobacteriacceae*; *Pseudomonas* spp.; *Staphylococcus* spp.; and *Streptococcus* spp. (SILVA *et al.* 1987).

d. Specific competition for nutrients or growth factors

Some bacteria (*Porphyromonas* and *Prevotella* species) require growth factors such as vitamin K. The growth of probiotic Bifidobacteria is stimulated by vitamin K as well (HOJO *et al.* 2007). Competition for the growth factor by *Bifidobacterium adolescentis* S2-1 resulted in the inhibition of growth of *P. gingivalis* and the decrease of vitamin K concentrations in the growth medium (HOJO *et al.* 2007).

The most widely used probiotics belong to the lactic acid bacteria (LAB) such as genus *Lactobacillus*. Lactobacilli comprise a large heterogeneous group of low-G+C gram-positive, nonsporulating, and anaerobic bacteria (CLAESSON *et al.* 2007). Taxonomically, the lactobacilli belong to the phylum Firmicutes, class Bacilli, order Lactobacillales, family *Lactobacillaceae*. In humans, the lactobacilli are part of the normal microbiota of the oral cavity, the gastrointestinal tract and the vagina. The same species can colonize both the oral cavity and the intestinal tract (MAUKONEN *et al.* 2008). In the oral cavity, the lactobacilli comprise less than 1% of the total cultivable microbiota such as *Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus rhamnosus* and *Lactobacillus salivarius* (AHRNE *et al.* 1998; SIMARK-MATTSSON *et al.* 2007). Most of these lactobacilli have a long history of safe use (produced in dairy industry) and are thus 'Generally Recognized As Safe' (GRAS). Although lactobacilli are regarded as a part of the normal oral microbiota, it has been shown that lactobacilli are associated with dental caries (LARMAS 1992).

According to sugar fermentation patterns, lactobacilli are divided into three groups – obligate homofermentative, obligate heterofermentative and facultative hetero-fermentative bacteria. The obligate homofermentative bacteria ferment sugars by glycolysis and produce lactate. The obligate heterofermentative bacteria use only the 6-phosphogluconate/phosphoketolase pathway and produce lactate and ethanol as end products. The facultatively heterofermentative bacteria are split into two categories – homofermentative and heterofermentative, consuming sugars hexoses and pentoses, respectively (AXELSSON 2004). In this thesis we used two probiotic LAB strains – *Lactobacillus salivarius* W24 and *Lactobacillus rhamnosus* GG, which belong to two different groups regarding their sugar fermentation.

Lactobacillus salivarius W24 – a homofermentative LAB

L. salivarius W24 is present in commercially available probiotic products (Winclove Bio Industries BV, Amsterdam, the Netherlands) aimed to restore the gastrointestinal microbial balance, *e.g.*, after antibiotic-associated diarrhea or traveler's diarrhea (KONING *et al.* 2008; TIMMERMAN *et al.* 2007). Moreover, W24 was shown superior in inhibiting coagulase negative staphylococci and *Staphylococcus aureus*, as well as other clinical pathogens such as *Klebsiella pneumoniae*, *E. faecalis* and *Escherichia coli* (TIMMERMAN *et al.* 2004; TIMMERMAN *et al.* 2007). W24 inhibited pro-inflammatory cytokine production in unstimulated peripheral blood mononuclear cells (PBMC) and had no negative selection criteria such as antibiotic resistance (TIMMERMAN *et al.* 2007). W24 is able to survive at a very acidic conditions (pH 2.5), as well as in the presence of bile and digestive enzymes (pancreatin and pepsin) (KONING *et al.* 2008). Although W24 is included in commercial probiotic products, the characteristics of this strain regarding oral health effects had not been addressed.

Lactobacillus rhamnosus GG – a facultatively heterofermentative LAB

L. rhamnosus GG (LGG) (ATCC 53103) (former name – *Lactobacillus acidophilus* GG or *Lactobacillus casei* GG) was originally isolated from a healthy human intestine in 1985 and named after the discoverers, Sherwood Gorbach and Barry Goldin. LGG colonies have a unique morphology (large, creamy white colonies that emit a buttery odor) when cultured on MRS agar, and this property facilitates their identification in mixed cultures (GOLDIN *et al.* 1992). The full characteristics of LGG are described elsewhere (DE KEERSMAECKER *et al.* 2006; HAUKIOJA *et al.* 2008b; NOSOVA *et al.* 2000; SILVA *et al.* 1987; SREEKUMAR *et al.* 2009). A brief summary of the main characteristics of this strain is provided below.

LGG ferments ribose, rhamnose, mannose, glucose, fructose, but does not ferment lactose, maltose, raffinose, or sucrose (SILVA *et al.* 1987). It has been shown that in the presence of glucose in the MRS medium, LGG grew and resulted in a fast decrease of the pH of the spent medium below pH 4 (HAUKIOJA *et al.* 2008b). Investigation of the spent MRS medium further revealed that LGG grew in MRS medium and then produced lactic acid and acetic acid (NOSOVA *et al.* 2000; SILVA *et al.* 1987). In the

presence of cystein or methione, LGG produces volatile sulphur compounds, *e.g.*, hydrogen sulphide (H_2S) and methanethinol (MeSH) (SREEKUMAR *et al.* 2009). Furthermore, LGG is able to survive under very acidic conditions (pH 1 - 2) (GOLDIN *et al.* 1992) and survival of LGG is enhanced in the presence of glucose (CORCORAN *et al.* 2005). In addition, LGG produces antimicrobial substances that can inhibit the growth of a wide range of bacteria (SILVA *et al.* 1987). These antimicrobial substances are in the acidic range. If pH was increased to nearly 7, the antimicrobial activity was no longer present (DE KEERSMAECKER *et al.* 2006; GOLDIN *et al.* 1992; SILVA *et al.* 1987). Examination of the spent culture MRS medium revealed components contributing to the antimicrobial activity, such as acetic acid, pyroglutamic acid, formic acid and lactic acid (DE KEERSMAECKER *et al*. 2006).

Previous studies on intestinal health revealed that LGG meets all criteria for an ideal probiotic strain for use in the dairy industry, *e.g.*, resistance to acid and bile (GOLDIN *et al.* 1992; JACOBSEN *et al.* 1999), attachment to human epithelial cells (ALANDER *et al.* 1997; ALANDER *et al.* 1999; ELO *et al.* 1991; TUOMOLA and SALMINEN 1998), colonization of the human intestine (SAXELIN *et al.* 1993; SAXELIN *et al.* 1991; SAXELIN *et al.* 1995), production of an antimicrobial substance (SILVA *et al.* 1987) and beneficial effects on human intestinal health (ARMUZZI *et al.* 2001; ARVOLA *et al.* 1999; GUANDALINI *et al.* 2000; VANDERHOOF *et al.* 1999) .

One of the mechanisms of action of probiotics in the gastrointestinal tract is based on the adherence to the intestinal mucosa and thereby inhibition of gut pathogens (SHERMAN *et al.* 2009). Similarly, oral probiotics should adhere to oral soft and hard tissues and prevent the adhesion or inhibit the growth of oral pathogens (DEVINE 2009). *In vitro*, LGG can adhere to the saliva-coated hydroxylapatite (HAUKIOJA *et al.* 2008a; STAMATOVA *et al.* 2009). However the reports on colonization of probiotic LGG in the oral cavity are contradictory: from no establishment at all (BUSSCHER *et al.* 1999) to colonization from few days (YLI-KNUUTTILA *et al.* 2006) to two weeks after discontinuation of the use of LGG-containing products (MEURMAN *et al.* 1994).

In vitro studies have shown that LGG has an effect on pathogenic species (HAUKIOJA *et al.* 2008a; MEURMAN *et al.* 1995; WEI *et al.* 2002). LGG inhibited the growth of *Streptococcus sobrinus* (MEURMAN *et al.* 1995). Likewise, LGG inhibited the adherence

of *S. mutans* and *S. sobrinus* on saliva-coated hydroxylapatite (HAUKIOJA *et al.* 2008a; WEI *et al.* 2002).

Only a few clinical studies on LGG and oral health effects that have been published suggest that LGG might have beneficial effects on oral health (TWETMAN and STECKSEN-BLICKS 2008). Children that were exposed to milk containing the probiotic LGG at their day-care centers for seven months showed less dental caries and lower mutans streptococci counts in saliva than children in the control group. This investigation suggested that the effectiveness of oral probiotics might vary by age (NASE *et al.* 2001). Adults consuming cheese with LGG for three weeks had less mutans streptococci counts in saliva but showed no effects on salivary *Candida* counts (AHOLA *et al.* 2002). In contrast, elderly people that were exposed to cheese containing mixture of LGG and other strains for four months, showed lower prevalence of oral *Candida* spp. (HATAKKA *et al.* 2007). On the contrary, the intervention by probiotics increased the salivary counts of lactobacilli and had no effects on mutans streptococci counts (MONTALTO *et al.* 2004). It has been suggested that a combination of the multiple species/strains could be more effective than only one specific bacterial strain (ZOPPI *et al.* 2001).

OBJECTIVE OF THE THESIS

In nature microorganisms do not live in isolation but are part of a complex community, where they share nutrients, metabolites, biochemical signals and genetic material. One important property of the community is that the survival fitness of the individual cell increases (*e.g.*, increased resistance to antimicrobials in medicine, to anti-fouling agents in pipe-lines and to preservatives in food processing). This in turn causes severe problems in health care and industry. Thus, our ultimate aim is to understand the species composition, separate activities and mutual interactions within such a community, as well as of their responses to changes induced in their environment.

One specific aim of this project was to develop a cost efficient, easy to handle and accurate technique that allows high throughput screening of the composition of dental plaque biofilms. To this end, we developed multiplex ligation-dependent

probe amplification (MLPA) method in order to monitor the oral microbial community composition. MLPA probes specific for 16S rDNA of a well documented and representative set of oral microorganisms (SOCRANSKY *et al.* 2004) were designed for community profiling. The probes were validated with samples obtained in *in vitro* experiments under controlled conditions with a predictable outcome.

As a particular application, but also second aim of the project, we undertook to understand the effects of a perturbation – two specific probiotic strains: *L. salivarius* W24 and *L. rhamnosus* GG (LGG) - on the composition, acidogenicity, as well as the cariogenic potential of the complex community (saliva-derived microcosm).

OUTLINE OF THE THESIS

In **chapter II** the ability of probiotic *L. salivarius* W24 to establish itself into the saliva-derived microbial communities was tested. Furthermore, the effects of the W24 on the compositional stability of oral microbial communities were assessed by denaturing gradient gel electrophoresis (DGGE).

In **chapter III** the applicability of multiplex ligation-dependent probe amplification (MLPA) for relative quantification of bacterial species in oral biofilms was assessed.

In **chapter IV** the interaction of probiotic *L. rhamnosus* GG (LGG) with the cariogenic bacterium *S. mutans* in dual species biofilms and the effects of LGG on cariogenic potential and microbial composition of saliva-derived microcosms were assessed and evaluated by the use of MLPA.

In **chapter V** we assessed the microbial composition of saliva and saliva-derived microcosms at the depth of 454 pyrosequencing and compared the output with the MLPA profiles.

This thesis is based on the following papers:

1. PHAM LC, VAN SPANNING RJ, RÖLING WF, PROSPERI AC, TEREFEWORK Z, TEN CATE JM, CRIELAARD W, ZAURA E (2009). Effects of probiotic *Lactobacillus salivarius* W24 on the compositional stability of oral microbial communities. *Arch Oral Biol* 54: 132-137.

2. TEREFEWORK Z, PHAM LC, PROSPERI AC, ENTIUS MM, ERRAMI A, VAN SPANNING RJ, ZAURA E, TEN CATE JM, CRIELAARD W (2008). MLPA diagnostics of complex microbial communities: relative quantification of bacterial species in oral biofilms. *J Microbiol Methods* 75: 558-565.

3. PHAM LC, HOOGENKAMP MA, EXTERKATE RA, TEREFEWORK Z, DE SOET JJ, TEN CATE JM, CRIELAARD W, ZAURA E (2011). Effects of *Lactobacillus rhamnosus* GG on saliva-derived microcosms. *Arch Oral Biol* 56: 136-147.

4. PHAM LC, BUIJS MJ, TEN CATE JM, CRIELAARD W, ZAURA E. Pyrosequencing analysis of human saliva and saliva-derived microcosms (Manuscript to be submitted to *Microbial Ecology*).