Microbial community interactions: effects of probiotics on oral microcosms

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MICROBIAL COMMUNITY INTERACTIONS

Effects of probiotics on oral microcosms

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. D.C. van den Boom

ten overstaan van een door het college voor promoties ingestelde

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Faculteit der Tandheelkunde

To my parents

To my husband and my daughter

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Contents

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*).

Chapter II - Effects of probiotic *Lactobacillus salivarius* W24 on the compositional stability of oral microbial communities

This chapter has been published as:

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.

ABSTRACT

Probiotics are microorganisms beneficial to gastro-intestinal health. Although some strains are also known to possess positive effects on oral health, the effects of most intestinal probiotics on the oral microflora remain unknown. We assessed the ability of the intestinal probiotic *Lactobacillus salivarius* W24 to incorporate into and to affect the compositional stability and cariogenicity of oral microbial communities. Microtiter plates with hydroxylapatite discs were incubated with W24 ("+W24") or without W24 ("–W24") and saliva from four individuals in plain ("–sucrose") or sucrose-supplemented ("+sucrose") medium. Biofilms were subjected to community profiling by 16S rDNA gene-based denaturing gradient gel electrophoresis (DGGE) after 72 h growth. Diversity (Shannon-Weaver index) and similarities (Pearson correlation) between biofilm communities were calculated.

Microcosms "+sucrose" were less diverse and more acidic than "–sucrose" microcosms ($p<0.001$). The effects of W24 on the community profiles were pH-dependent: at pH 4 ("+sucrose"), the respective "+W24" and "–W24" microcosms differed significantly more from each other than if the pH was ~7 ("–sucrose"). The pH of "+W24/+sucrose" microcosms was lower ($p<0.05$) than the pH of the microcosms supplemented with sucrose alone ("–W24/+sucrose").

Although not able to form a monospecies biofilm, *L. salivarius* W24 established itself into the oral community if inoculated simultaneously with the microcosm. In the presence of sucrose and low pH, W24 further lowered the pH and changed the community profiles of these microcosms. Screening of probiotics for their effects on oral microbial communities allows selecting strains without a potential for oral health hazards.

INTRODUCTION

Probiotics are defined as live microorganisms which when administered in adequate amount confer a health benefit on the host (FAO/WHO 2006). The effective use of probiotics has been reported in treatment of intestinal diseases such as inflammatory bowel disease, antibiotics-associated diarrhea, and irritable bowel

syndrome (ARMUZZI *et al.* 2001; MACFARLANE and CUMMINGS 2002; VAN SANTVOORT *et al.* 2008), as well as non-gastrointestinal diseases, such as atopy, respiratory infections, vaginitis and hypercholesterolaemia (ZUCCOTTI *et al.* 2008). Probiotic supplements are generally regarded as safe because the microorganisms they contain are identical to those found in the human gastrointestinal and vaginal microflora. Although probiotics are administered orally by ingestion, so far the studies on these microorganisms with respect to oral health are scarce (TWETMAN and STECKSEN-BLICKS 2008) and their effects on oral microbial ecology remain unknown.

The most abundantly used probiotic strains are of the genus *Lactobacillus*. Lactobacilli are commensal lactic acid producing bacteria with high aciduric potential. A probiotic lactobacilli strain, *Lactobacillus salivarius* LS1952R was found to be highly cariogenic in rats (MATSUMOTO *et al.* 2005), while the oral administration of probiotics containing seven *Lactobacillus* species significantly increased the salivary counts of lactobacilli in healthy adults and had no effect on *Streptococcus mutans* (MONTALTO *et al.* 2004). In contrary to these results, there are clinical studies on probiotics and oral health suggesting that probiotic bacteria may have beneficial effects on dental health (TWETMAN and STECKSEN-BLICKS 2008). Children that were exposed to milk containing the probiotic *Lactobacillus rhamnosus* GG for seven months, showed less dental caries and lower mutans streptococci counts than children in the control group (NASE *et al.* 2001). In addition, a study on an adult population also found reduced salivary mutans streptococci, in this case after 3-week ingestion of *Lactobacillus reuteri* ATCC 55730 (CAGLAR *et al.* 2006).

Among the various intestinal probiotic lactobacilli strains tested, *Lactobacillus salivarius* W24 was superior in inhibiting coagulase negative *Staphylococcus* and *Staphylococcus aureus*, as well as other clinical pathogens such as *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Escherichia coli* (TIMMERMAN *et al.* 2004; TIMMERMAN *et al.* 2007). Moreover, W24 inhibited pro-inflammatory cytokine production in unstimulated peripheral blood mononuclear cells and had no negative selection criteria such as antibiotic resistance (TIMMERMAN *et al.* 2007). This strain is included in commercially available probiotic products (Winclove Bio Industries BV, Amsterdam, the Netherlands) used to restore the gastrointestinal

microbial balance, *e.g.,* after antibiotic-associated diarrhea or traveler's diarrhea (KONING *et al.* 2008; TIMMERMAN *et al.* 2007). *L. salivarius* belongs to obligatory homolactic lactobacilli that produce only lactic acid during glucose fermentation. We hypothesized that addition of *L. salivarius* to oral microbial community may increase cariogenicity of dental plaque biofilm.

Our aim was to test the ability of intestinal probiotic *L. salivarius* (strain W24) to establish itself into the saliva-derived microbial communities. Furthermore, we aimed to assess the effects of W24 establishment on the compositional stability and cariogenicity of the microbial communities derived from individual salivas.

MATERIALS AND METHODS

The inoculum for microcosms

The use of human saliva was approved by institutional review board. Stimulated saliva was collected during parafilm chewing from four healthy adults with caries experience in the past and that no use of antibiotics within last three months. The donors were asked not to brush their teeth for 24 h and to abstain from any food or drink intake for at least 2 h before donating saliva. During collection, saliva was kept on ice. After that, the saliva was filtered through sterilized glass-wool and diluted in glycerol (final concentration 30%). The mixture of saliva and glycerol was aliquoted in 2 mL sterile tubes, and stored at - 80 °C. One of the frozen aliquots was processed in advance of the experiment to quantify the bacteria by colony counting on blood agar plates after anaerobic incubation (80% N_2, 10% CO_2 and 10% H_2) at 37 °C for 48 h. An inoculum of 10^6 colony forming units (cfu)/mL was subsequently used in microcosm experiments.

Biofilm growth conditions and harvesting

The growth medium comprised artificial saliva medium described by McBain *et al* (MCBAIN *et al.* 2005), and contained mucin (type II, porcine, gastric), 2.5 g/L; bacteriological peptone, 2.0 g/L; tryptone, 2.0 g/L ; yeast extract, 1.0 g/L; NaCl, 0.35 g/L, KCl, 0.2 g/L; $CaCl_2$, 0.2 g/L; cysteine hydrochloride, 0.1 g/L; haemin, 0.001 g/L; vitamin K1, 0.0002 g/L), pH 7. Sterilized hydroxylapatite (HA) discs (Ø: 10.6 mm)

were put into the wells of polystyrene, 24-well flat-bottomed microtiter plates. Each well was filled with 2 mL of growth medium either with or without 0.2% (v/v) sucrose supplementation. As inoculum the saliva-glycerol stock was added (10^6 cfu/mL) and the plates were incubated anaerobically at 37 °C for 72 h. The medium was refreshed every 24 h.

After growth the HA discs were removed from the wells, put into tubes with 1 mL of cysteine peptone water (CPW) and vortexed at maximum speed for two minutes. The biofilm samples were then centrifuged at 16,100 x *g* for one minute. The samples were processed for denaturing gradient gel electrophoresis (DGGE). The pH of the spent medium was measured by pH electrode (PHM 220 Lab pH Meter, Meterlab®, Radiometer Analytical SAS, France).

Ability of *L. salivarius* strain W24 to establish into microcosms

A freezer stock (overnight culture + 30% glycerol) of *L. salivarius* W24 was streaked onto a MRS agar plate and grown at 37 °C anaerobically for 48 h. One colony of W24 from the agar plate was used to inoculate 10 mL of artificial saliva medium with 0.2% sucrose and grown anaerobically at 37 °C for 16 h.

The optimal concentration of W24 to inoculate the microcosm was determined by inoculating a series of concentrations of W24 (from 10^2 - 10^8 cfu/mL) with 10^6 cfu/mL of saliva in sucrose-supplemented artificial saliva medium and incubating the microcosms for 72 h as described above. A concentration of 10^4 cfu/mL W24 was found to give the DGGE profiles consisting of multiple bands including a distinct W24 band, while higher concentrations yielded a single dominant W24 band on the DGGE gel, and lower concentrations showed no W24 band at all (data not shown). The concentration of 10^4 cfu/mL of W24 was chosen as the optimal concentration to inoculate the microcosms described below.

Saliva-derived microcosms were grown anaerobically, in the medium supplemented with 0.2% sucrose, on the HA discs at six different conditions: the microcosm alone (a control), the probiotic strain W24 introduced once (at 0 h, 24 h or 48 h), twice (at 24 h and 48 h) or thrice (at 0 h, 24 h and 48 h) into the microcosm. The microcosms were harvested after 72 h and were processed for DGGE.

To control for pH-induced effects resulting from the metabolism of sucrose, the same conditions as above were tested in a medium supplemented with PIPES buffer (Sigma-Aldrich, USA).

The effects of sucrose and the probiotic *L. salivarius* strain W24 on microcosms derived from four individual salivas

Saliva from four healthy adults was used to inoculate microcosms. Two independent experiments per saliva were performed with triplicate samples per experimental condition. The microcosms were grown at four experimental conditions: 1) in a plain medium without W24; 2) in a plain medium with W24 added at 0 h; 3) in a sucrose-supplemented medium without W24; 4) in a sucrose-supplemented medium with W24 added at 0 h. The microcosms were harvested after 72 h and processed for DGGE. The pH of the spent medium was determined after 24 h, 48 h (data not shown) and 72 h.

Denaturing gradient gel electrophoresis (DGGE)

DNA was extracted with the DNeasy blood and tissue kit following the instructions of the manufacturer (Qiagen, Germany). PCR was carried out in a 25 μL (total volume) mixture containing 0.4 μM Muyzer primer F357-GC, 0.4 μM primer R518 (Table 1) (MUYZER *et al.* 1993), each deoxynucleoside triphosphate at a concentration of 0.4 mM, 10 μg of bovine serum albumin (Biolabs, 10 mg/mL), 1 x Taq buffer, 0.25 U of Taq enzyme, and 1 μL of undiluted DNA template. Amplification was performed with a PCR machine (Tprofessional thermocycler, Biometra, Germany) as follows: 94 °C for four minutes, followed by 35 cycles of 94 °C for 0.5 minute, 54 °C for one minute, and 72 °C for one minute, and a final elongation at 72 °C for five minutes.

DGGE was performed with the Bio-rad DCode system. The PCR product was loaded onto 1-mm-thick 8% (wt/vol) polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) gels containing a 35% to 60% linear denaturing gradient; 100% denaturant was defined as 7 M urea and 40% (vol/vol) formamide. The outer two lanes of each gel were not used. The samples were distributed randomly among

different gels. To aid in the conversion and normalization of the gels, a marker consisting of 7 reference strains was added at both sides of each gel, as well as after every four samples. The marker consisting of a mixture of 7 reference strains (*Porphyromonas gingivalis, L. salivarius* W24*, Agregatibacter actinomycetemcomitans, Streptococcus mutans, Veillonella parvula, Peptostreptococcus assacharolyticus* and *L. reuteri*), for which the V3 region (*Escherichia coli* positions 357 to 518) was amplified.

The gels were electrophoresed in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM Na-EDTA; pH 8) at 200 V and 60 °C for 4 h. The gels were stained in 1 x TAE buffer containing 1 μg/mL of ethidium bromide and were recorded with a charge-coupled device camera system (The imager; Appligen, Illkirch, France).

Table 1. Primers used in this study.

Name	**Primers (5′ – 3′)**
GC clamp	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCCC
F357*	GC CGC CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGG CCT ACG GGA GGC AGC AG
R518*	ATT ACC GCG GCT GCT GG

* - Numbers 357 and 518 indicate nucleotide position in *E. coli*.

Analysis of the community profiles

Microbial diversity is the variation in microbial species in an ecosystem. To estimate microbial diversity, diversity indices are calculated statistically. A frequently used index is the Shannon Weaver index of general diversity (VERSEVELD and RÖLING 2004). Here, it takes into account the number of DGGE bands and the relative contribution of each band to the whole set of bands. Band positions were assigned manually. The individual bands and their intensities in the community profiles were determined using GelCompar. The Shannon Weaver index (H′) was calculated

as follows: $H' = - \Sigma p_i \log p_i$ where p_i is the relative contribution of band *i* to the whole set of bands in a track calculated as $p_i = n_i/N$; n_i is the height of a peak in a densitometric curve and *N* is the sum of all peak heights. A higher H' indicates a higher diversity (VERSEVELD and RÖLING 2004).

Similarities between DGGE profiles were calculated using GelCompar software (version 4.0, Applied Maths, Sint-Martens-Latem, Belgium) by Pearson product-moment correlation coefficient and visualized using the Unweighted Pair Group Clustering Method with Arithmetic Averages (UPGMA) (VERSEVELD and RÖLING 2004). The average similarity values per growth condition from two independent experiments were calculated. UPGMA is a clustering method used to construct a tree from a matrix of pairwise distances between samples. The first step in UPGMA is identification of the pair of samples with the smallest distance between them. The branch point is estimated as half the distance between the two samples. The two samples are then defined as a cluster and the matrix is recalculated with the first two samples combined. This process is repeated with the number of entries in the matrix reduced by each time from the root by adding clusters defined in each of the matrices.

Statistical analyses

The Statistical Package for the Social Sciences (SPSS version 14) was used to perform statistical analyses. For all tests, triplicate samples from two independent experiments were averaged into a single value per individual saliva and growth condition. The normality of data was confirmed with Shapiro-Wilk normality test ($p>0.05$), the homogeneity of variance – with Levene's test ($p>0.05$). The effects of the experimental conditions on the pH and the diversity values of the microcosms were calculated using one-way ANOVA and the Bonferroni post-hoc test. The correlation between the pH and the diversity data of 72-h old microcosms was calculated using Pearson's correlation analysis. The significance level of all tests was 0.05.

RESULTS

Ability of *L. salivarius* strain W24 to establish into microcosms in the presence of sucrose

In preliminary experiments where the ability of W24 to grow in a biofilm was tested we found that this strain is not able to form a biofilm when incubated as a monoculture in the microtiter plate model.

The pH of spent medium of microcosms grown without PIPES supplement was pH 4.0 (SD 0.08), while with PIPES the pH was 6.8 (SD 0.02). W24 established itself into the biofilm community irrespective of the pH (Figure 1). However, W24 was able to establish itself into the microcosm only if W24 was added simultaneously with the inoculum (T = 0 h) (Figure 1). Microcosms that were inoculated with W24 once or twice at a later time point (T = 24 h or T = 48 h) did not show a distinct *L. salivarius* band on the DGGE gel.

The impact of W24 on the community profiles was pH-dependent. In the samples that were grown with W24 but without PIPES, some distinct DGGE bands either appeared (Figure 1, band "a") or disappeared (Figure 1, band "b") compared to the microcosms grown without W24, while no changes in the band profiles due to W24 were observed in microcosms supplemented with PIPES.

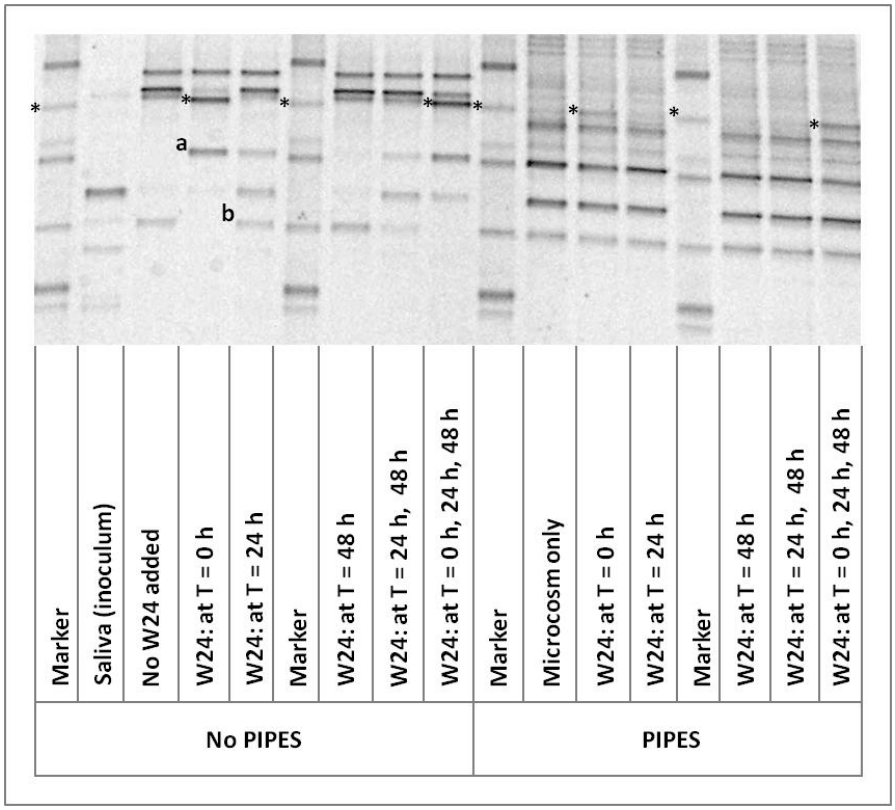

Figure 1. The effects of the inoculation time and frequency on the establishment of the *L. salivarius* W24 into the microcosms grown for 72 h, as revealed by DGGE profiling of amplified bacterial 16S rDNA gene fragments. Biofilms were grown in either medium supplemented with 0.2% sucrose alone (No PIPES) or in medium supplemented with 0.2% sucrose and PIPES (PIPES). Six conditions per growth medium were compared: 1) no W24, 2) W24 inoculated simultaneously with saliva into the microcosm at the start of the experiment (W24: at T = 0 h), 3) W24 inoculated into 24 h-old microcosms (W24: at T = 24 h), 4) W24 inoculated into 48-h old microcosms (W24: at T = 48 h), 5) W24 inoculated twice – into 24- and 48-h old microcosms (W24: at T = 24 h, 48 h), 6) W24 inoculated thrice – simultaneously with saliva at the start of the experiment and into 24- and 48-h old microcosms (W24: at T = 0 h, 24 h, 48 h).

* - the band corresponding to the *L. salivarius* W24 position in the marker line.

a, b - examples of individual bands affected by the presence of W24.

Effects of sucrose and *L. salivarius* W24 on microbial communities derived from four individual salivas

The biofilm communities in microcosms with or without sucrose were unique for each individual (data not shown). However, W24 was able to establish itself into all microcosms irrespective of individual or the presence of sucrose. Figure 2 shows the average pH of the spent medium and the average diversity of the biofilm communities, derived from four individual saliva donors after 72-h growth. There was a significant correlation between the diversity of DGGE profiles and the pH of the spent medium ($p<0.001$; Pearson correlation coefficient 0.893). Sucrose had a significant effect on both pH and the diversity of the microcosms. The pH and the diversity of the sucrose-exposed microcosms were significantly lower ($p<0.001$) than the respective values of the microcosms grown in the plain medium. W24 lowered the pH of sucrose-exposed microcosms for additional 0.3 pH unit, which was significantly lower ($p=0.046$) than the pH of the microcosms exposed to sucrose alone.

Sucrose alone had a strong effect on the community profiles in all four individual microcosms: the sucrose-exposed samples of each individual clustered separately from the respective samples grown in plain medium (avg. similarity 23%, SD 12.2). The effects of W24 on community profiles of all four individual microcosms were dependent on the presence of sucrose (Figure 3). In the absence of sucrose, W24-supplemented microcosms remained rather similar to the microcosms without W24 added (avg. similarity 56%, SD 12.3). When W24 was added in the presence of sucrose, the microcosms showed significantly reduced similarity (avg. similarity 27%, SD 12.4) and a changed band pattern of the DGGE profiles (Figure 1).

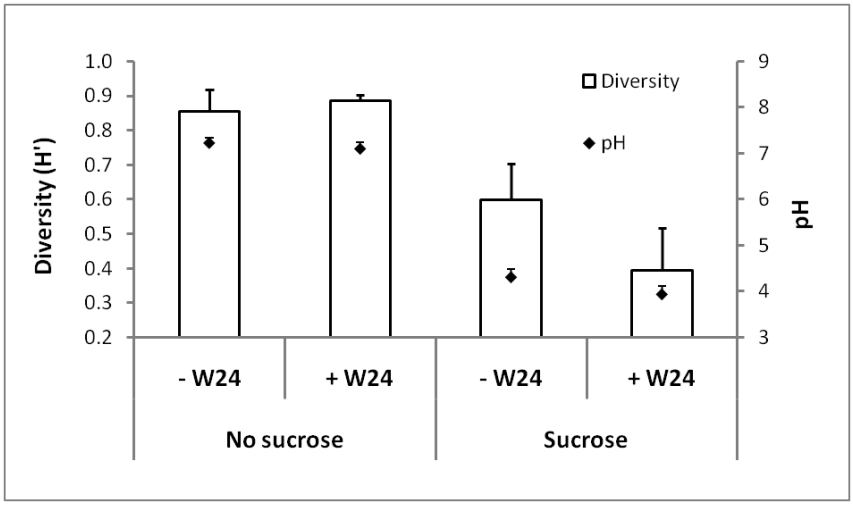

Figure 2. Shannon-Weaver Index of Diversity (H') and the pH of the spent medium of the 72-h old microcosms grown in the medium with or without 0.2% sucrose and with (+W24) or without *L. salivarius* W24 (-W24) added to the inoculum. The medium was refreshed after 24 h and 48 h. The values are average from the duplicate experiments (N=3 per experiment) with four microcosms derived from individual saliva donors. Error bars indicate standard deviations.

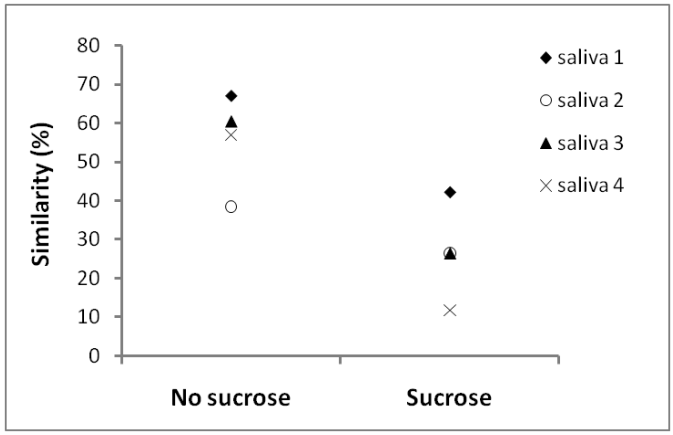

Figure 3. The effects of sucrose on the similarity between the DGGE profiles of the 72-h microcosms with *L. salivarius* W24 and the microcosms without W24 added per individual saliva used to inoculate the microcosms. The similarity values were obtained from Pearson correlation after UPGMA analysis of the DGGE profiles. The values are average from two independent experiments with triplicate samples per growth condition. 'No sucrose' – the microcosms grown in the plain medium; 'Sucrose' – the medium was supplemented with 0.2% sucrose.

DISCUSSION

We studied the effects of two ecological perturbations – sucrose and a probiotic strain – and their mutual interaction on the stability of oral microcosms. Under specific growth conditions (low pH and concurrent inoculation with the microcosm), intestinal probiotic *Lactobacillus salivarius* W24 was able to affect the biofilm ecology of the microcosms derived from saliva of different individuals and appeared to have a cariogenic potential.

We employed sucrose as a well-known modulator of the ecology of oral communities: microbial shifts due to sucrose metabolism and resulting low pH have been demonstrated for defined microbial consortia (BRADSHAW *et al.* 1989) and complex saliva-derived microcosms (FILOCHE *et al.* 2004; FILOCHE *et al.* 2007b). We also observed clear microbial shifts in our study, which provides further support to the ecological plaque hypothesis proposed by Marsh (MARSH 1994). In our model, the pH of the spent medium decreased from pH 7 to about pH 4 in the presence of sucrose, while it remained around pH 7 when sucrose was not added to the growth medium. The selective pressure of the low pH on microbial community was visualized in changed patterns of the DGGE fingerprints and summarized in decreased diversity of the profiles. Interestingly, a strong positive correlation between the pH and the diversity of the microcosms was found. More diverse microbial communities have been shown to be associated with caries-free individuals compared to caries-active subjects (LI *et al.* 2007; LI *et al.* 2005).

L. salivarius W24 is one of the probiotic strains available in multispecies commercial probiotic products aimed at the improvement of gastrointestinal microbial balance (KONING *et al.* 2008; TIMMERMAN *et al.* 2004; TIMMERMAN *et al.* 2007). Assessment of the potential of W24 to establish into the microcosms showed that this strain, although unable to form a biofilm while growing in a monoculture, was able to establish into saliva-derived communities irrespective of the pH of the medium. Interestingly, the prerequisite for successful establishment of W24 was the concurrent inoculation of the saliva microorganisms and W24. This suggests that, *in vivo* situation, this strain might be able to establish on dental surfaces within newly developing dental biofilm if exposed to the oral cavity right after tooth

brushing. This also proposes that besides the availability of surface for attachment, an interaction with other microorganisms or their products was mandatory for the biofilm formation of W24.

Furthermore, for W24 to have an impact on the ecology of the microbial community, a low pH was necessary. Only in the presence of un-buffered growth medium and sucrose, changes in the DGGE profiles of the W24-supplemented microcosms were observed. Two possible mechanisms might explain the observed effects of W24 on the biofilm communities. Firstly, the addition of W24 lowered pH even further than sucrose alone and thus might have selected the most aciduric members in the microcosms. Secondly, a common feature of probiotic strains is the release of bacterocins or bacterocin-like substances (CORR *et al.* 2007; JOERGER and KLAENHAMMER 1986; KLAENHAMMER 1993; VIGNOLO *et al.* 1993) that negatively affect other, potentially pathogenic microbial species. In our preliminary studies, W24 did not affect the biofilm formation of *S. mutans* in a dual species model (unpublished findings), while it does inhibit clinical isolates associated with infections elsewhere in the human body, such as *Escherichia coli*, *Enterococcus faecalis, Staphylococcus aureus* and *Klebsiella pneumoniae* (TIMMERMAN *et al.* 2007).

It has been shown that individual saliva 'begets unique microcosms' (LEDDER *et al.* 2006) and each individual community may respond in its own manner to ecological perturbations (FILOCHE *et al.* 2007b). To increase the relevance of our findings, we assessed the effects of W24 on microcosms derived from saliva of four individuals and repeated each experiment twice. Even though each individual community and the microcosm derived from it is unique (LEDDER *et al.* 2006; RASIAH *et al.* 2005), sucrose together with W24 introduced the same phenomena – reduced diversity and lowered pH – in all individual microcosms in a reproducible manner. On the other hand, the appearance or disappearance of individual DGGE bands due to establishment of W24 in the presence of sucrose, was highly individual (data not shown) and is attributable to inter-individual differences in microbial community composition.

Our results showed that addition of an aciduric and obligatory homolactic microorganism, *L. salivarius* W24, to oral microcosms in the presence of sucrose

lowered the pH and thus might increase the cariogenic potential of the oral microbial community. The model described here is an oversimplification of the complex dynamic community interactions occurring in the oral cavity. Our approach could be used as a high throughput screening of existing and newly developed probiotics with regard to their effects on the oral microbial communities. Further clinical testing would be necessary to exclude the potential risks, *e.g.*, increased acid formation rather than inhibiting cariogenicity, for the oral health of the commercially available intestinal probiotic products.

In conclusion, we showed that the intestinal probiotic *L. salivarius* W24 was not only able to establish into saliva-derived microcosms, but also affected the compositional stability and reduced the pH of these microcosms. Screening of intestinal probiotic strains for their effects on oral microbial communities could improve the selection of beneficial strains for the host as a whole.

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Chapter III - MLPA diagnostics of complex microbial communities: relative quantification of bacterial species in oral biofilms

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ABSTRACT

A multitude of molecular methods are currently used for identification and characterization of oral biofilms or for community profiling. However, multiplex PCR techniques that are able to routinely identify several species in a single assay are not available. Multiplex ligation-dependent probe amplification (MLPA) identifies up to 45 unique fragments in a single tube PCR. Here we report a novel use of MLPA in the relative quantification of targeted microorganisms in a community of oral microbiota. We designed nine species specific probes for *Actinomyces gerencseriae, Actinomyces naeslundii, Actinomyces odontolyticus, Candida albicans, Lactobacillus acidophilus, Rothia dentocariosa, Streptococcus mutans, Streptococcus sanguinis* and *Veillonella parvula*; and genus specific probes for selected oral streptococci and lactobacilli based on their 16S rDNA sequences. MLPA analysis of DNA pooled from the strains showed the expected specific MLPA products. Relative quantification of a serial dilution of equimolar DNA showed that as little as 10 pg templates can be detected with clearly discernible signals. Moreover, a 2 to 7% divergence in relative signal ratio of amplified probes observed from normalized peak area values suggests MLPA can be a cheaper alternative to using qPCR for quantification. We observed 2 to 6 fold fluctuations in signal intensities of MLPA products in DNAs isolated from multispecies biofilms grown in various media for various culture times. Furthermore, MLPA analyses of DNA isolated from saliva obtained from different donors gave a varying number and intensity of signals. This clearly shows the usefulness of MLPA in a quantitative description of microbial shifts.

INTRODUCTION

The human oral cavity harbors a multifarious group of microorganisms that forms a complex community and occupies diverse specific niches and microenvironments. The resident oral microflora is sustained in an apparent state of balance or microbial homeostasis, once microorganisms are established in the mouth (MARSH 2003a). Environmental perturbations change the dynamic ecology of the resident, evoking microbial shifts in terms of concentrations and activities, which at times may result

in the development of cariogenic and periodontal diseases (MARSH 2000; MARSH 1994). Dental plaque, a surface bound community which is regarded as a biofilm is of particular importance in the ecology of the oral microbiota. The formation of plaque or growth of biofilm on dental surfaces by rapid colonization of bacteria follows a particular microbial succession, which is largely dependent on the host's genotype, quality of the immune system, diet, general hygiene and health conditions (MARSH 2003a; PALMER *et al.* 2007). Growing in a biofilm gives certain advantages to the resident microflora (SCHEIE and PETERSEN 2004). The biofilm life style enables bacteria to develop mechanisms that minimize the effect of antimicrobials and the human immune defense system (GILBERT *et al.* 1997; MAH and O'TOOLE 2001).

About half of the more than 700 bacteria species inhabiting the oral cavity are culturable, though this assumption is made by studying mainly sub-gingival samples (AAS *et al.* 2005; PRATTEN *et al.* 2003). Traditional culturing methods and biochemical assays thus do not allow to fully characterizing the inhabitants of the oral cavity. Instead, molecular techniques provide the ideal tools for identification and characterization of bacteria that are hitherto undiscovered and most of the traditionally uncultivable bacteria. Current determination of the microbial etiology of dental diseases which utilizes both the culturing and molecular methods is far from being adequate (MARSH 2003b). Similarly, quantification of the bacteria in biofilms is a daunting task. The change in the dynamics of the resident flora due to changes in the environment poses a challenge for accurate determination of the spatial and temporal abundance of the species. *In vitro* models show ways of evaluating the shift in populations associated with the onset of common oral diseases (DALWAI *et al.* 2006). Real-time PCR based methods such as the Taq-man system, qPCR and checkerboard DNA-DNA hybridization are shown to quantify oral bacteria in biofilms (DALWAI 2007; MARTIN *et al.* 2002; NADKARNI *et al.* 2002; SOCRANSKY *et al.* 2004; SUZUKI *et al.* 2004a; SUZUKI *et al.* 2005; SUZUKI *et al.* 2004b). Arrays of molecular methods are used for identification and characterization of oral biofilms or for community profiling. Various multiplex PCR techniques targeting the 16S rDNA gene (TRFLP, DGGE, LH-PCR), southern blotting, genomic fingerprinting using random PCR are so far the most widely used. However, these

commonly used methods all have their shortcomings. For instance, southern blotting is cumbersome and time-consuming; PCR generates false positives; restriction site based techniques are ponderous to perform; genomic fingerprinting generates arbitrary data and a metagenomics approach is time consuming and requires expensive equipments. Thus, there is a need to develop a simpler, faster and cost effective method for a relative quantification of a selected set of bacteria in multispecies consortia at different time points and at different nutritional and environmental conditions.

The multiplex ligation-dependent probe amplification (MLPA) method (SCHOUTEN *et al.* 2002) is widely used to identify genes in various human genetic disorders that result in different diseases. This method is proven to identify these genetic alterations whether they are a result of point mutations, insertions, deletions, duplications or recombination events (HOGERVORST *et al.* 2003; STERN *et al.* 2004; VOLIKOS *et al.* 2006; VORSTMAN *et al.* 2006; WILTING *et al.* 2006). Notwithstanding the complex process of designing probes, the elegance and simplicity of this method makes it applicable to any type of DNA. Recently an MLPA based assay which is able to identify 15 respiratory viruses has been described (REIJANS *et al.* 2008). The only MLPA based assay on bacteria we are aware of is the recent characterization of *Mycobacterium tuberculosis* using drug resistance markers for identification (BERGVAL *et al.* 2008). A ligase chain reaction (LCR) (WIEDMANN *et al.* 1994) based method, which is similar to MLPA, was shown to detect multiple blood-borne bacterial pathogens in a single assay (PINGLE *et al.* 2007). The aim of this study was to investigate the use of MLPA to screen the composition and dynamics of complex microbial communities, using oral biofilms as an example, and to develop an easy to perform and cost effective multiplex assay for the relative quantification of a selected set of bacteria in a single reaction. Our report here shows the application of MLPA in simultaneously identifying targeted oral bacteria in a specific manner from pure cultures, consortia of known species, as well as DNA obtained from biofilm and saliva samples. We also show the use of MLPA in detecting the shifts in bacterial composition in biofilms when the ecology of the oral microbiota is changed due to changes in the environment.

MATERIALS AND METHODS

Sample collection and preparation

A panel of eight oral bacterial species was chosen for the MLPA assay (Table 1). These species were selected because they are frequently found in dental biofilms and the presence of most of these is associated with dental caries (FILOCHE *et al.* 2007b). Biofilms were grown in a constant depth film fermentor (CDFF) using saliva from a single source as inoculum as described (DENG *et al.* 2005; WILSON 1999) or in a consortium in 20 mL Bijou tubes. Stimulated saliva was collected from six healthy subjects. The saliva collected was filtered through a sterilized glass-wool and diluted in 20 mL glycerol in a sterile tube, and then stored at - 80 °C. To inoculate the CDFF, thawed saliva was mixed together with 200 mL of artificial saliva medium (mucin (type II, porcine, gastric), 2.5 g/L; bacteriological peptone, 2.0 g/L; tryptone, 2.0 g/L; yeast extract, 1.0 g/L; NaCl, 0.35 g/L, KCl, 0.2 g/L; $CaCl_2$, 0.2 g/L; cysteine hydrochloride, 0.1 g/L; haemin, 0.001 g/L; vitamin K_1, 0.0002 g/L) (MCBAIN *et al.* 2005). The mixture of saliva and artificial medium was pumped into the CDFF at the flow rate of 2.4 mL per minute in conventional mode of the CDFF as originally designed (WILSON 1999). The inoculation was settled for 1 h, and then the medium was supplied at flow rate 0.3 mL per minute for 3 hours by using the back-and forth mode of the CDFF as described (DENG *et al.* 2005). The sucrose pulse was supplied 8 times per day at flow rate 1.2 mL per minute for 5 minutes and then continued with pumping the medium. The biofilms grown in the CDFF were collected after 14 days.

In a parallel study, a biofilm was formed in 20 mL Bijou tubes (Grenier Bio-one, Frickenhausen, Germany) from a consortium of eight species (*A. gerencseriae, A. naeslundii, A. odontolyticus, C. albicans, L. acidophilus, S. mutans, S. sanguinis* and *V. parvula*). The strains were grown on blood agar first and then half a loop of the pure culture was transferred to 2 mL artificial saliva medium (MCBAIN *et al.* 2005) and grown at 37 °C anaerobically overnight. 1.5 mL of the fully grown culture from each species was then added to sterile 20 mL Bijou tubes and mixed vigorously. Then, 0.6 mL of the consortium mix was grown in 20 mL Bijou tubes containing three different media (10 mL) - artificial saliva alone, artificial saliva supplemented with 0.2% sucrose and artificial saliva supplemented with 0.2% sucrose and PIPES

(50 mM). The medium was refreshed after 24 h and 48 h incubation. Biofilm was collected 7 h, 24 h and 48 h after incubation at 37 °C.

DNA isolation

DNA from individual strains was extracted from fully grown cultures with a concentration of 10^6 - 10^7 colony forming units (cfu) per mL using the FastDNA® SPIN® Kit (For Soil) (Q-BIOgene) according to the manufacturers' instruction. Community DNA from multispecies saliva or CDFF grown biofilms samples was obtained after bead beating followed by using DNeasy Blood & Tissue Kit (Qiagen). More specifically, biofilms were resuspended in 1 mL ATL buffer (Qiagen) and transferred to a bead beating vial containing 0.5 gram of 0.1 mm sterilized glass beads (Biospec products, Inc.). Bead beating was done at 5.5 m/s for 30 sec. (Fast prep Qbiogene). The mix was then centrifuged at 14,000 x g for 5 minutes. From this step onwards we followed the DNeasy Blood & Tissue Kit instruction manual. We also isolated DNA from the eight species biofilm/consortium (*A. gerencseriae, A. naeslundii, A. odontolyticus, C. albicans, L. acidophilus, S. mutans, S. sanguinis* and *V. parvula*) sample grown in a single tube the same way as from saliva and CDFF biofilms. All DNAs were treated with RNAse to avoid RNA interference with probe hybridization.

MLPA probe design

The MLPA probes we designed target the various regions of the 16S rDNA molecule (Table 1), except for the *S. sanguinis* probe, which is based on 23S rDNA. Full MLPA probe amplification is only possible after successful hybridization of the two oligonucleotides half-probes adjacent to each other on the target DNA and subsequent ligation to one another. The ligation of the two probes is very specific and can only occur when the probes are exactly bound to their specific complementary target. Any sequence dissimilarities, *e.g.*, single nucleotide polymorphisms (SNPs) at the ligation site, will prevent proper ligation and will result in the absence of a peak signal.

Initially we designed synthetic probes following the guidelines of MRC-Holland (Amsterdam, the Netherlands). Briefly, the criteria for designing probes require a minimum length difference between probes - 4 nucleotides. Tm of each hybridizing sequence, Left Probe Oligonucleotide (LPO) or Right Probe Oligonucleotide (RPO): ≥70°C. ΔG (secondary structure): ≥ 0. LPO with preferably a maximum of 2 G/C directly adjacent to the ligation site and preferably a maximum of 3 G/C directly adjacent to the primer were chosen. No more than 7 nucleotide overlap was allowed between probes to avoid competition. Genus and species specific probes were designed manually using a global alignment of 930 sequences from oral samples obtained from the ribosomal database project RDP database. The probe match program from RDP (http://rdp.cme.msu.edu/probematch/search.jsp) was used to determine the specificity of the probes. In addition, we used the BLAST algorithm to inspect the uniqueness of our selected probe sequences. Both the LPOs and RPOs were synthesized by Biolegio (Nijmegen, the Netherlands). Once we determined that specific signals are obtained from our probes, the RPOs were manufactured by MRC-Holland using the M13-cloning based production strategy as described previously (SCHOUTEN *et al.* 2002).

Table 1. Strains and probes used in this study.

Strains	Probe*	MLPA product size
Actinomyces naeslundii (ATCC 12104)	CGAACGGTGAAGGGGCCTGCTTTTGT**GGGTCCTGGAT GAGTGGCGAACGGGTGAGTAACACGTG** (74 – 159)	336
Actinomyces gerencseriae (ATCC 23860)	GTGCCGGTTGTCCTTCGGA**GACGGAGGGTCCTCCCTT TGCGGGGGTCGGTTCACAG** (1074 – 1131)	202
Actinomyces odontolyticus (ATCC 17929)	CGGGTTGTGAACCTCTTTCGCTCAT**GGTCAAGCCGCA ACTCAAGGTTGTGGTGAGGG** (485 – 546)	364
Candida albicans (CBS 8575)	CGAATCGCATGGCCTTGTGCTG**GCGATGGTTCATTCA AATTTCTGCCCTATCAACTTTC** (261 – 308)	191
Lactobacillus acidophilus (ATCC 4356)	GAGATTCGCTTGCCTTCGCAGGCTTGC**TCCTCGTTGTA CTGTCCATTGTAGCACGTGTGTAG** (1316 – 1368)	341
Rothia dentocariosa (ATCC 17931)	CGAAGAACCTTACCAAGGCTTGACATATACTGGACTG CGT**CAGAGATGGCGTTTCCCTTCGGGGCTGGTATAC AG** (1048 – 1131)	171
Streptococcus mutans (ATCC 25175)	GCTATGGCTCAACCATAGTGTGCTCTGGAAACTGTCTG **ACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGT GTA** (675 – 753)	301
Veillonella parvula (ATCC 10790)	CCGTGATGGGATGGAAACTGCTG**ATCTAGAGTATCG GAGAGGAAAGTGGAATTCCCATGT** (689 – 750)	252
Streptococcus sanguinis (ATCC 10556)	CTTGTGTTATGGCCACTCTAACCCGGTAG**GTTTATCAT CTACGGAGACAGTGTCTGACGGGC**	126
streptococci common**	CACTGTGACGGTATCTTACCAGAAAGG**GACGGCTAA CTACGTGCCAGCAGCCGCGGTAATA** (535 – 595)	152
lactobacilli common***	CGTAAACGATGAATGCTAGGTGTTGGA**GGGTTTCCGC CCTTCAGTGCCGCAGCTAACG** (881 – 941)	326
Universal 1	GTGAATACGTTCCCGGGCCT**TGTACACACCGCCCGTC ACACCACGAGAGTTTGTAACA** (1458 – 1512)	237
Universal 2	CGGAGGAAGGTGGGGATGACGTCA**AATCATCATGCC CCTTATGACCTGGGCTACACACGTGCTA** (1258 – 1321)	185
L. fermentum (ATCC14931)		
L. plantarum (SA-1)		
L. salivarius (ATCC 11741)		
S. anginosus (ATCC 33397)		
S. mitis 2 (SK 149)		
S. parasanguinis (M1021)		
S. sobrinus (OIHI)		

* - Only hybridizing sequences included. Left probe oligonucleotides (plain), right probe oligonucleotides (**bold**). Numbers in brackets indicate nucleotide positions in *E. coli.*

** - streptococci common probes identify: *S. anginosus*, *S. gordonii*, *S. oralis*, *S. intermedius*, *S. mitis*1, and *S. mitis*2.

*** - lactobacilli common probes identify: *L .casei*, *L. paracasei*, *L. rhamnosus*, and *L. fermentum.*

MLPA reaction

MLPA reactions on bacterial DNA were performed with a slight modification of the standard protocol for human DNA. Routinely 50 – 500 pg of DNA from pure cultures or 500 pg – 10 ng from saliva and biofilm samples was used. All the MLPA reagents were provided by MRC-Holland (Amsterdam, the Netherlands). The whole MLPA protocol was performed in T1-gradient thermo cycler (Biometra GmbH, Göttingen, Germany) according to the standard MLPA protocol http://www.mlpa.com/pages/support_mlpa_protocolspag.html. The PCR products were separated by capillary electrophoresis using either the CEQ 800 genetic analyzer (Beckman instruments) (Cy5 labeled primers were used) or the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) (primers were labeled with FAM).

Probe specificity and validation of signal quantification

We performed experiments where either DNA from a single species or pooled DNA of equimolar proportions from all or part of the strains under study is hybridized against a varying combination of probe mixes containing either single pairs of (LPO and RPO) or mixed pairs with varying combinations.

Template DNAs (100 pg) were spiked with varying amounts of foreign DNA obtained from human and other related bacteria against which only the universal probes but not the species specific ones are hybridized. This was to test the minimum amount of DNA detectable when standard conditions are used and to assess risk of contamination. In addition, to check the possible cross-reactivity of probes, species specific probes were tested on DNA obtained from closely related species with a high degree of homology with the 16S rDNA sequences. The following cross-reactivity tests were done. *S. mutans* probe was tested against *Streptococcus parasanguinis*, *Streptococcus sanguinis*, *Streptococcus mitis* 2, *Streptococcus angiosus* and *Streptococcus sobrinus*; the *L. acidophilus* probe was tested against *Lactobacillus salivarius*, *Lactobacillus fermentum* and *Lactobacillus plantarum*; the *Actinomyces* species probes were tested against each other.

Serial dilution of pooled DNA (5 ng – 30 pg), from strains whose probes are based on 16S rDNA, were used as template to test the validation of quantifiable signals obtained from the reactions. A control plasmid (33 zmol) that contains the target sequences of two probes producing 141 and 237 bp length fragments was added to the reactions as an internal control. These control probes, unlike the DQ controls are subject to all parameters affecting the result of the MLPA reaction. They are used to test efficiency of hybridization, ligation and PCR amplification of ligated probes. They are also used as internal standards to normalize signal intensities for relative quantification. The resulting signals (peak area) were normalized as described previously (SCHOUTEN *et al.* 2002). The reactions were done in triplicate; the intensity of signals was normalized by dividing signal intensity of each probe to the sum of all signals from the control probes in each dilution series. This relative signal was in turn divided by the signal ratio of its own control probe deduced from the average value of triplicate runs. The statistical significance of the data was investigated by calculating the standard deviation for the triplicate in each data series as well as performing a general linear model repeated measures test (GLM-RM) in SPSS (version 14).

Community profiling with DGGE

We employed DGGE using the primers described by Muyzer *et al* (MUYZER *et al.* 1993) to investigate the microbial shifts occurring when biofilms were produced under different environmental conditions. We tested a biofilm (consortium) containing eight species grown in a single tube where samples were grown for 7 h, 24 h and 48 h in three different media. DGGE was performed using the Bio-Rad D-Code system (Bio-Rad, Herculas, CA) as described by Muyzer *et al* (MUYZER *et al.* 1993). The gel image was processed and quantification of bands was performed using the GelCompar II version 4.0 software package (Applied Maths, Kortrijk, Belgium). The relative band intensities were calculated using the markers as a reference and then compared with those obtained from the MLPA experiments.

RESULTS

MLPA

The initial tests of MLPA using synthetic probes showed that specific signals from each sample can be obtained. However, the signals contained shoulder peaks which made size calling tedious and problematic. After confirming that MLPA works using synthetic probes, we finally reverted to using the M13-derived probes. This strategy enabled us to add stuffer sequences with varying lengths at the right probe oligonucleotide (RPO). This in turn allows the production of up to 45 probes which contain sufficient size differences to be separated by other means, for instance gel electrophoresis, rather than by capillary electrophoresis. In addition we found that the peaks generated by these probes were sharper and contained almost no shoulder peaks (Figure 1).

We were able to generate specific amplified probe fragments using the probes that were designed for these strains. A mixed sample containing equimolar DNA pooled from the eight strains in the study produced all the specific MLPA products, as well as the common probes directed to a group of streptococci and lactobacilli, and the control "universal" probes designed to detect most of the oral bacteria (Figure 1). Only a single specific MLPA product is obtained when a template DNA from a single species is hybridized with a probe mix that contained all of the probes (Figure 2). The cross reactivity tests on the *S. mutans*, *L. acidophilus* and the *Actinomyces* spp. did not produce any MLPA product other than those that are generated from universal or from group specific probes. We also observed that as little as 10 pg of template DNA can be detected with discernible signal, depending on the fidelity or reproducibility of probes, which is dependent on their length, folding capacity, Tm, sequence uniqueness and the oligo quality.

The effect of variations in copy number and probe fidelity was investigated by performing MLPA on serially diluted equimolar amount of DNA pooled from the target species (Figure 3). The relative ratio of normalized signals is shown in Table 2. A ratio value of 1 is thus an indication of similar amount of template or copy number. In a typical MLPA reaction on human DNA cut off values higher than 1.3 or lower than 0.7 are used to indicate copy number increase or gene duplication

and/or deletion. The normalized values show that at each concentration the relative signal intensity remains unchanged. The p value for the GLM-RM was >0.05, the standard deviations ranged between 0.007 – 0.09.

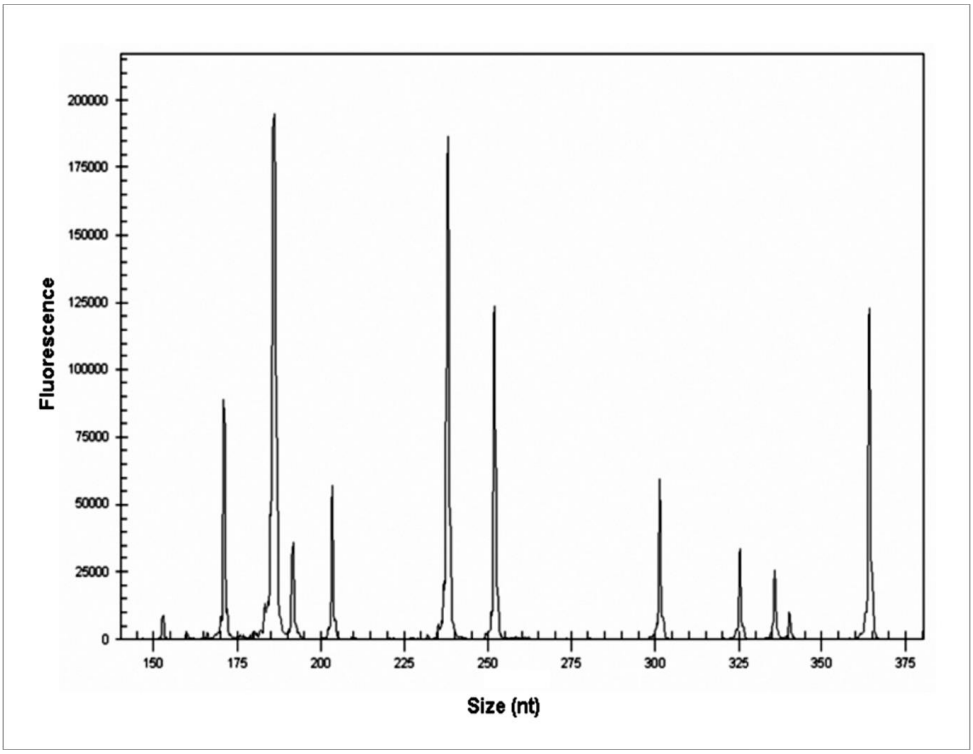

Figure 1. Electropherogram showing signals obtained from a mixed sample. Equimolar amounts of DNA pooled from the eight species used as template. Fragment sizes (nt) correspond to: 152 = selected streptococci, 171 = *R. dentocariosa*, 185 = Universal probe 1, 191 = *C. albicans*, 203 = *A. gerencseriae*, 237 = Universal probe 2, 252 = *V. parvula*, 301 = *S. mutans*, 326 = lactobacilli, 336 = *A. naeslundii*, 341 = *L. acidophilus*, 364 = *A. odontolyticus*. The universal probes target most of the oral bacteria.

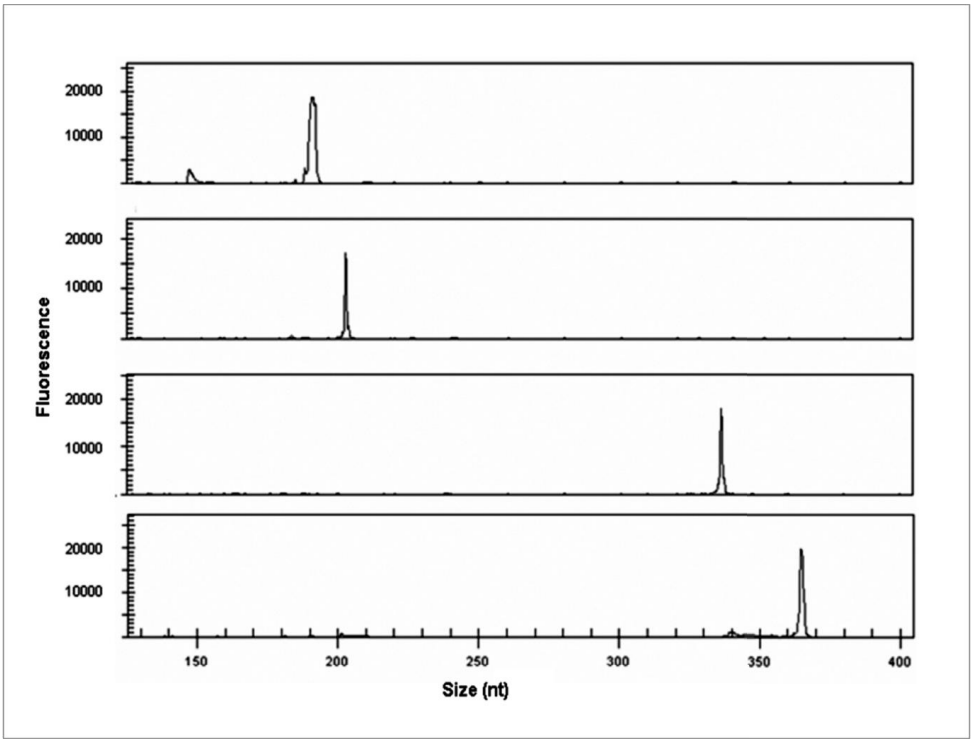

Figure 2. Electropherogram showing single peaks generated by MLPA. Template DNA from individual species is used in the reaction. The MLPA reaction included all the 12 probes designed. Fragment sizes (nt) correspond to: 191 = *C. albicans*, 203 = *A. gerencseriae*, 336 = *A. naeslundii*, 364 = *A. odontolyticus*.

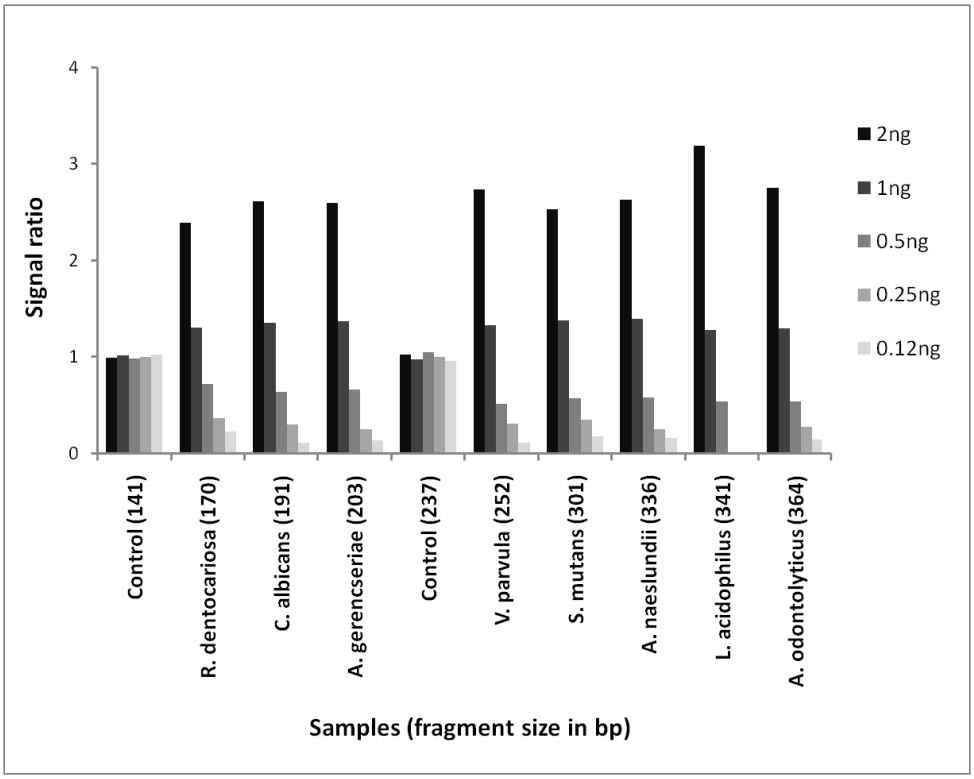

Figure 3. Relative quantification of MLPA fragments. Two fold serial dilution of DNA from each species and control plasmid (33 zmol) were used as template in each dilution series. Fluorescence data from control probes (141 nt and 237 nt) were used to normalize the data obtained from the samples. The reaction was done in triplicate. Intensity of the signal from each sample in a dilution series is divided by the sum of the signals from the control probes in the respective series. The resulting ratios were in turn divided by the average signal from the triplicate runs. The p value for the GLM-RM was >0.05, the standard deviations ranged between 0.007 – 0.09.

Table 2. Relative signal ratio of probes observed from peak area values. The values are normalized by dividing signal intensities of individual probes to the sum of signal intensities from all probes. The resulting ratio is again divided by the average of the triplicate values obtained from each probe. The p value for the GLM-RM was >0.05, the standard deviations ranged between 0.007 – 0.09.

Species	Fragment	Template DNA concentrations							
	Size (nt)	5 ng	2 ng	1 ng	500 pg	250 pg	125 pg	60 pg	30 pg
R. dentocariosa	171	0.647	0.778	0.938	0.935	0.934	1.092	1.035	0.959
C. albicans	191	1.004	1.003	1.024	0.972	0.972	1.018	0.998	1.017
A. gerencseriae	203	1.016	1.002	1.052	1.016	0.959	0.966	0.951	0.981
V. parvula	252	0.981	0.977	0.865	0.959	1.033	1.010	1.004	0.959
S. mutans	301	0.998	1.023	1.090	1.087	1.065	0.976	1.018	1.001
A. naeslundii	336	1.024	1.012	1.061	1.042	1.008	0.991	1.029	1.051
L. acidophilus	341	1.010	1.031	1.062	1.022	0.978	0.963	0.994	1.210
A. odontolyticus	364	0.987	0.966	0.889	0.949	1.012	1.006	0.975	1.002

Application of MLPA on community DNA

With the selected set of MLPA probes, we observed that the MLPA profiles obtained from DNA isolated from different saliva donors were distinct. Moreover, the peak sizes, which indicate the relative abundance of detected bacteria in each sample, were also clearly different (data not shown). DNA obtained from CDFF grown biofilm also showed a distinct pattern. The eight species in the mixed species biofilm or consortium that can be detected by our probes were traced back accurately. The strains abundance in the biofilm; as shown in fluctuation of their numbers due to changes in environment, incubation time and the type of media used was also apparent in the variable intensity of signals obtained (Figure 4). Microbial shift was discernible from the increase and decrease of signal intensities from all samples that were grown in different media. A microbial shift was also

observed in the biofilm samples grown in the CDFF (Figure 5). This shift was most pronounced in sucrose supplemented medium where a sharp increase in *A. odontolyticus* was observed. MLPA detected *S. mutans* in the CDFF samples grown in sucrose supplemented medium but not in the inoculum saliva and the medium without sucrose additive. Apparently, *S. mutans* was not present in detectable amount in this saliva sample. Changes were also evident in numbers of *R. dentocariosa* and *A. naeslundii*. These species were not detected anymore in the resulting biofilm samples. Reciprocally fluctuating streptococci and *Actinomyces* species in oral biofilms were reported in other studies as well (DALWAI *et al.* 2006; MOORE and MOORE 1994).

Comparison of DGGE with MLPA

In general, we found that the community compositions as deduced from DGGE profiles were in agreement with those from MLPA profiles. Relative band intensities of the samples whose products were visible on the gel corresponded to signal intensities obtained from MLPA profiles. However, the sensitivity of DGGE was lower than that of MLPA as judged by the observation that *A. gerencseriae* and *A. naeslundii* bands were not visible on the DGGE gel, but they were detected by MLPA analyses. In addition, DGGE seems to be more prone to PCR bias than MLPA. The fluctuation of *S. mutans* and *L. acidophilus* in the consortium was not detected by DGGE.

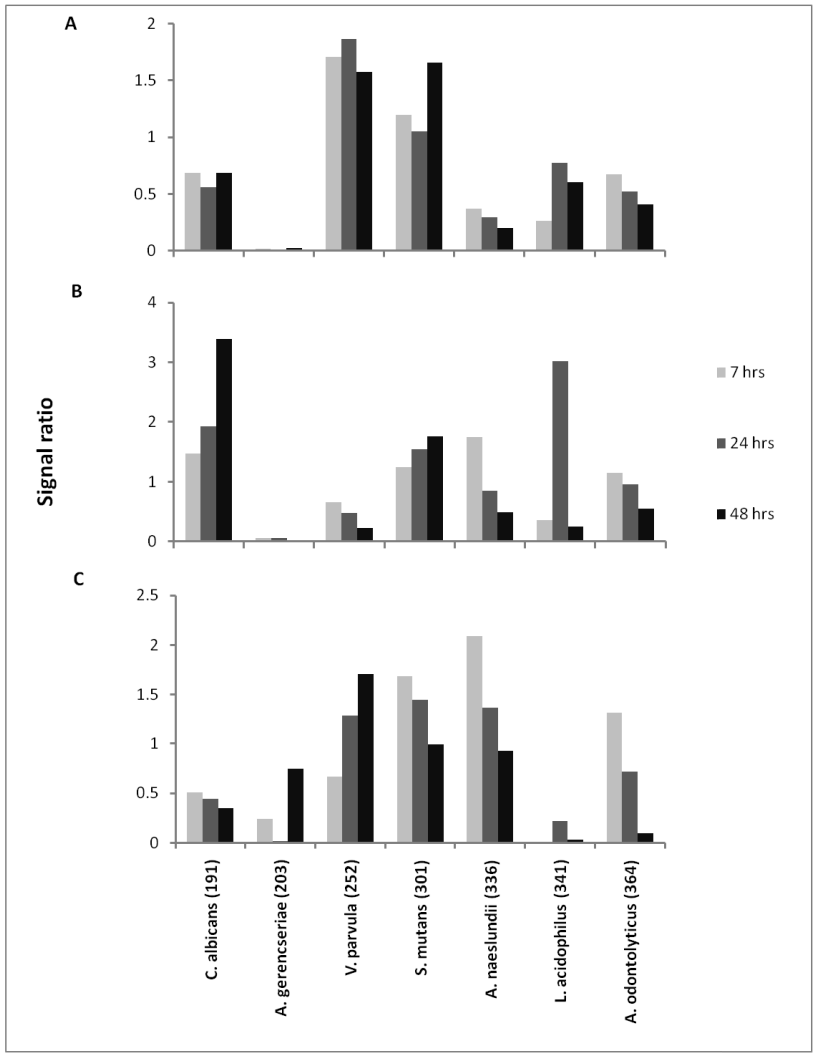

Figure 4. Variation in signal intensities of a biofilm (consortium) composed of eight species. The biofilm was grown in three different media for three different incubation times: (**A**) Biofilm grown in artificial saliva + sucrose + pipes medium; (**B**) Biofilm grown in artificial saliva + sucrose medium; (**C**) Biofilm grown in artificial saliva medium alone. Fragment sizes correspond to the following microorganisms: 191 = *C. albicans*, 203 = *A. gerencseriae*, 252 = *V. parvula*, 301 = *S. mutans*, 336 = *A. naeslundii*, 341 = *L. acidophilus*, 364 = *A. odontolyticus*. Signal ratios were obtained by dividing the signal intensity of each peak with the sum of all signals in the same series. These ratios were then divided by the respective average ratios of the triplicate runs. The p value for the GLM-RM was >0.05.

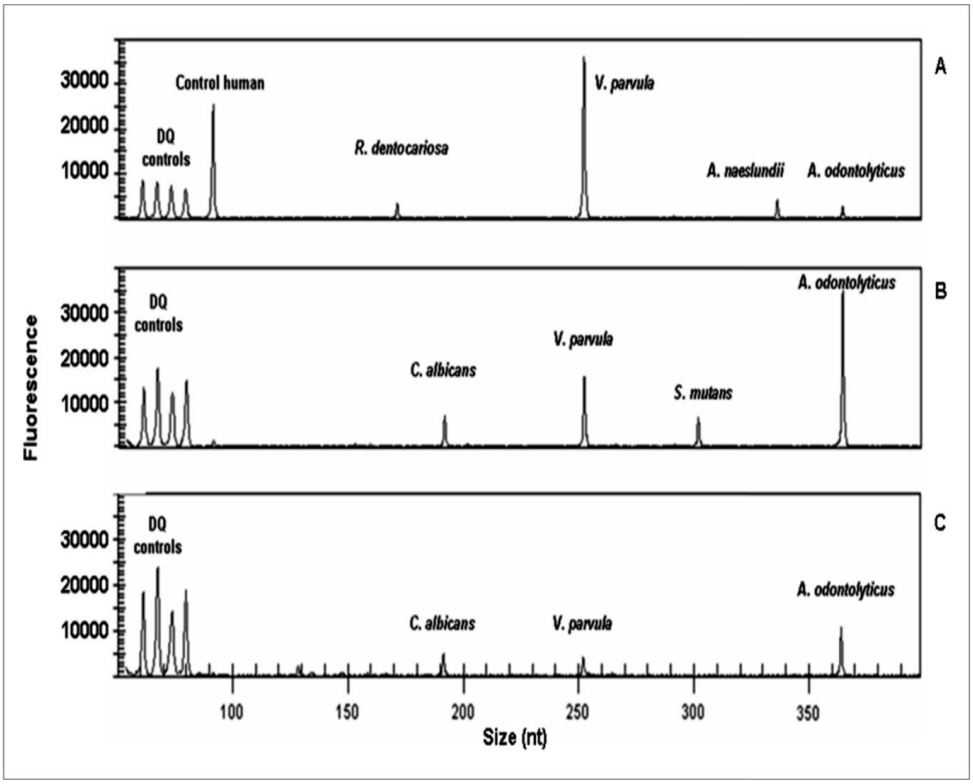

Figure 5. MLPA profiles obtained from (**A**) stimulated human saliva that was used to inoculate the CDFF; (**B**) CDFF microcosm biofilm grown in sucrose-supplemented medium; (**C**) CDFF microcosm grown in medium with no sugar supplement. The detection of control probe for human DNA in the saliva sample shows the DNA extracted from saliva contained human DNA.

DISCUSSION

We designed a set of MLPA target sequences for the species we selected in this study, all of which yielded probe fragments of the expected size following the PCR based amplification method during MLPA. These probes tested on DNAs isolated from pure bacterial cultures, and communities contained in crude saliva as well as CDFF biofilms and consortium produced the expected specific signals with 100% accuracy. No cross reactivity was observed between probes. Variations in the MLPA profiles and peak areas obtained from different saliva donors confirmed that the

composition of the microflora in the saliva of each individual is unique and characteristic for the donor. The growth of *S. mutans* which is commonly implicated in cariogenicity is shown to be affected by sucrose supplement (CURY *et al.* 2001). The detection of this species in the sucrose supplemented experiment but not in the crude saliva or the artificial saliva medium seems to corroborate this. About 6,000 copies of target DNA is required in a standard MLPA reaction (SCHOUTEN *et al.* 2002). Pre amplification of target molecules followed by a proper dilution can be used to enhance the sensitivity of detecting targets that are found in very small amount (REIJANS *et al.* 2008). MLPA is a PCR-based assay hence it is subject to PCR bias. We observed over saturation of the PCR reaction when a template is present in abundance, compromising the sensitivity of the assay for relative quantification. The biofilms from the eight species consortium grown in Bijou tubes also show variation in incubation time and the medium used. The overall divergence between conditions varied from 10 – 40%. This percentage indicates that a single parameter change could trigger dramatic changes in the composition of the microflora. This was more evident in our study particularly when the artificial saliva medium was supplemented with sucrose.

These results show the advantage of MLPA in investigating the dynamics of oral biofilm and accurate assessment of the spatial and temporal variations in the oral microbial communities. Understanding this dynamics and being able to monitor it would have a significant benefit in disease management (LILJEMARK *et al.* 1997; MARSH 1994). Fluctuations in composition and abundance of oral bacterial species were investigated using FISH (AL-AHMAD *et al.* 2007), quantitative real-time PCR (LYONS *et al.* 2000), Taq-man real-time PCR (SUZUKI *et al.* 2005) and competitive PCR (RUPF *et al.* 1999). However, these studies focus on a few species and do not accurately reflect the situations in biofilms, albeit the species are considered to play important role in the onset of various dental problems. The simultaneous detection and relative quantification of up to 45 specific amplicons using MLPA provides an excellent opportunity to investigate the microbial dynamics as well as determining the presence and abundance of oral pathogens. Our results that showed an overall 2 – 7% divergence in relative signal ratio of amplified probes from normalized peak area values indicate that MLPA can be a cheaper alternative to real-time PCR

assays. We compared the MLPA profiles obtained with the DGGE patterns and observed that MLPA is more sensitive. Comparison of MLPA with monoplex real-time PCR shows a comparable sensitivity (REIJANS *et al.* 2008). Reducing the hybridization time to two hours did not have a significant effect on the sensitivity of the MLPA reaction. Therefore, the whole reaction can be performed within a working day. In addition, automation of the whole procedure and a high throughput detection of several samples in a single assay are feasible.

The data presented here show the applicability of MLPA in identification of bacterial species from a community. In general, this accurate multiplex assay can be used as a rapid diagnostic tool in identification of a pathogen and for monitoring microbial dynamics in complex bacterial communities. The advantage of MLPA lies in identifying up to 45 unique sequences in a single tube PCR reaction by using only a single primer pair. In addition, it is fast and easy to perform. Quantification of products where there is no large variation between copy numbers in samples is also possible. Comparison of MLPA to other widely used molecular methods such as FISH and STR confirmed that it is reliable, accurate fast and cost effective (FERNANDEZ *et al.* 2005; PALOMARES *et al.* 2006). As was reported on Mycobacteria samples (BERGVAL *et al.* 2008), MLPA can also be used to screen clinical isolates and characterize pathogens. The 16S rDNA gene in bacteria contains about 1,500 nucleotides, of which conserved, variable and hypervariable regions span the whole molecule (WOESE 1987). No other gene has been as extensively sequenced and studied for phylogenetic and taxonomic purposes as 16S rDNA. The outcome of the plethora of 16S rDNA based studies, a large sequence database (MAIDAK *et al.* 2001; MAIDAK *et al.* 1997), is often used for determining the taxonomic positions of both cultivable and un-cultivable bacterial strains. Bacterial genomes contain up to 15 copies of the 16S rDNA gene (FOGEL *et al.* 1999). These different copies are shown to exhibit varying degree of redundancy and heterogeneity (ACINAS *et al.* 2004). The variable regions of 16S rDNA are ideal for highly specific MLPA probe design. However, the small size of this gene presents a problem in designing an extensive MLPA-probe library especially from species or strains that have very similar sequences. Nevertheless, using unique MLPA probes targeting genus and species specific signature sequences in this molecule enable to identify bacterial species in a

community. Other phylogenetic marker sequences, such as from 23S rDNA (as used for *S. sanguinis* in this study) and other commonly used house-keeping genes, can also be used for MLPA probe design provided their specificity to the species can be determined by comparing with adequate number of closely related sequences.

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Chapter IV - Effects of *Lactobacillus rhamnosus* GG on saliva-derived microcosms

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ABSTRACT

The probiotic strain *Lactobacillus rhamnosus* GG (LGG) is shown to hamper the presence of mutans streptococci in saliva and may have positive effects on oral health. We investigated the effects of LGG on the cariogenic potential and microbial composition of saliva-derived microcosms. Single and dual species biofilms of LGG and *Streptococcus mutans*, and saliva-derived microcosms with or without LGG were grown in an Active Attachment Biofilm model. The microcosms were grown on bovine dentin/enamel discs in the presence or absence of sucrose (suc+/suc-). The presence of LGG was determined by multiplex ligation-dependent probe amplification (MLPA) and real-time PCR. Mutans streptococci (MS) and total viable counts, pH of the spent medium, capacity of lactate formation and integrated mineral loss in dentin was assessed. MLPA was used for identification and relative quantification of 20 oral microorganisms in the microcosms. Principal Component Analysis was applied to MLPA data.

LGG inhibited the growth of *S. mutans* in dual species biofilms and did not affect the pH. LGG established in saliva-derived microcosms and reduced MS counts significantly, but did not affect pH or dentin demineralization. Simultaneous growth of the microcosms with LGG under heavy cariogenic conditions (suc+) introduced a compositional shift in the microbial community. The CFU, real-time PCR and MLPA data correlated significantly.

We conclude that LGG established into and inhibited the growth of MS in complex saliva-derived biofilms, but this had no significant effect on cariogenic potential of the microcosms. This suggests that other microorganisms besides MS were responsible for increased cariogenicity of sucrose-exposed biofilms.

INTRODUCTION

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO 2006). Probiotics are used in commercial products and they are considered to be good for intestinal

health (MARTEAU *et al.* 2002). However, studies assessing the effects of intestinal probiotics on oral health are scarce.

There is some evidence (TWETMAN and STECKSEN-BLICKS 2008) that probiotic bacteria may hamper the presence of mutans streptococci (AHOLA *et al.* 2002; CAGLAR *et al.* 2006; NASE *et al.* 2001; NIKAWA *et al.* 2004) and yeasts (AHOLA *et al.* 2002; HATAKKA *et al.* 2007) in saliva, while others reported that administration of probiotic lactobacilli resulted in an increase of salivary lactobacilli counts with no effect on mutans streptococci (MONTALTO *et al.* 2004). There is an obvious lack of evidence on caries inhibitory effects, except for a single clinical study on very young children (NASE *et al.* 2001). That study was carried out on children of 1–6 years of age, and the experimental group was served milk containing *Lactobacillus rhamnosus* GG at their daycare centers 5 days per week for 7 months. The best effect was found in the group of 3- to 4-year olds in which 6% of children developed new caries lesions during the study period in the experimental group compared with 15% in the un-supplemented milk group.

L. rhamnosus GG (LGG) has been shown to inhibit the adhesion/growth of *S. mutans* (HAUKIOJA *et al.* 2008a; KOLL *et al.* 2008; WEI *et al.* 2002) and to promote co-aggregation between lactobacilli/mutans streptococci (LANG *et al.* 2010). Besides *S. mutans*, LGG inhibited *Streptococcus sobrinus* (MEURMAN *et al.* 1995), *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis* and *Prevotella intermedia* (KOLL *et al.* 2008) in simple inhibition assays. However, there are no studies that assess the effects of LGG on complex oral microbial communities and their cariogenic properties.

In this study we aimed to assess the interaction of LGG with *S. mutans* both in dual species biofilms and in saliva-derived microcosms and to investigate effects of LGG on cariogenic potential of these biofilms.

MATERIALS AND METHODS

Dual and single species biofilms

Dual and single species biofilms were grown on hydroxylapatite (HA) discs (10.6 mm diameter) using an Active Attachment Biofilm (AAB) model (EXTERKATE *et al.* 2010). The model consists of a custom-made stainless steel lid with nylon clamps that can accommodate 24 substrata and fit into a 24-well plate. The HAP discs were positioned vertically and fitted into the wells without touching the wall of the well. After assembling the lid and the specimens, the model was autoclaved.

A freezer stock (overnight culture with glycerol, 30%) of *L. rhamnosus* GG ATCC 53103 (LGG) or *S. mutans* UA159 was streaked onto a MRS agar plate or BHI agar plate, respectively, and grown at 37 °C anaerobically (80% N_2, 10% CO_2 and 10% H_2) for 48 h. One colony of LGG or *S. mutans* from the agar plate was used to inoculate 10 mL of MRS or BHI medium, respectively, and grown anaerobically at 37 °C for 16 h.

The growth medium for dual and single species biofilms comprised artificial saliva medium (MCBAIN *et al.* 2005) supplemented with 0.2% (v/v) sucrose (PHAM *et al.* 2009). The wells of a polystyrene 24-well plate (Multiwell plates, Greiner Bio-One, Alphen a/d. Rijn, the Netherlands) were filled with 1.5 mL of growth medium. As inoculum the overnight culture of each species was added (10^6 cfu/mL). The biofilms were grown at five experimental conditions: 1) LGG alone; 2) *S. mutans* alone; 3) LGG and *S. mutans* added simultaneously at the time of inoculation (T = 0 h); 4) LGG added at T = 0 h and *S. mutans* added at T = 24 h; 5) *S. mutans* added at T = 0 h and LGG added at T = 24 h. The dual species biofilms were harvested after 48 h, the single species biofilms – after 24 and 48 h.

The model was incubated anaerobically at 37 °C for 48 h. The medium was refreshed at 8, 24 and 32 h. Each experiment was performed with triplicate samples and repeated thrice. The pH of the spent medium was measured by a pH electrode (PHM 220 Lab pH Meter, Meterlab®, Radiometer Analytical SAS, France).

After 48 h, the HAP discs were detached from the lids, put into 1.8 mL of PBS and sonicated (amplitude 40, pulser 1 sec, duration 2 min) (Sonics, Vibracell™, Sonics &

Materials Inc., Connecticut, USA). Serial dilutions of each sample were plated on BHI agar plates using a Spiral Plater (Eddy Jet, IUL, S.A, Spain) for determination of LGG and *S. mutans*. The plates were incubated anaerobically at 37 °C for 72 h.

Microcosm biofilms derived from individual salivas

Saliva-derived microcosms were grown on enamel-dentin discs using the AAB model as described above. The use of human saliva was approved by institutional Review Board. The enamel-dentin discs were cut perpendicularly to the labial surface of the crowns of bovine incisors with water-cooled trephine burr. The enamel and dentin surface was polished with abrasive paper strips (240 grit, 400 grit, 600 grit; Carbimet®, USA). Two notches were made at each side of the cylinder using a rotating diamond disc (Diaflex H 345/220; Horico, Germany); a piece of an orthodontic wire was placed in each notch and wrapped around the specimen. The loop of the wire was fixed to the nylon clamp in the lid of the AAB model and the model with specimens autoclaved.

The inoculum was prepared as described previously (PHAM *et al.* 2009) and contained 10^6 cfu/mL of total viable cell counts. The growth medium for microcosms comprised of artificial saliva medium (MCBAIN *et al.* 2005). The microcosms were grown at four experimental conditions: 1) in plain medium without LGG; 2) in plain medium with LGG; 3) in sucrose-supplemented medium; 4) in sucrose-supplemented medium with LGG. The microcosms were harvested after 72 h. In conditions 2 and 4, 10^6 cfu/mL of LGG was added to the medium at the time of inoculation (T = 0 h). Medium was refreshed at 8, 24, 32, 48 and 56 h. Every microcosm experiment was performed with triplicate samples and repeated twice. The pH of the spent medium was measured as described above.

Acid production assay

At 72 h, the lid containing the dentin-enamel discs and adherent biofilms was transferred to a sterile plate containing 1.5 mL cysteine peptone water (CPW) with 0.2% glucose. The model was incubated anaerobically for 3 h at 37 °C. After 3-h incubation, the incubation fluid was transferred into Eppendorf tubes and

incubated at 80 °C for 5 min to stop metabolism. The amount of L-lactic acid in the incubation fluid was determined enzymatically using a colorimetric assay (GUTMANN and WAHLEFELD 1974) (Spectrophotometer, Spectra Max M2; Molecular devices, USA).

Biofilm harvesting

After the acidogenicity test the specimens with adherent biofilms were detached from the lids as described above for the single and dual species biofilms. Serial dilutions of each biofilm sample were plated on TSAB and SAB agar plates using the Spiral Plater for enumeration of total viable bacteria and mutans streptococci counts, respectively. The plates were incubated anaerobically at 37 °C for 48 h. The biofilm samples were then centrifuged at 16,100 x *g* for 1 min and pellets stored at - 80 °C until DNA extraction.

Assessment of mineral loss

The effects of the microcosms on mineral content of the dentin part of the specimens were assessed by transversal microradiography (LAGERWEIJ *et al.* 1994). Two 200-µm-thick adjacent sections were cut from the centre of each cylinder perpendicularly to the surface. The sections together with the aluminium stepwedge were radiographed under wet conditions (VAN STRIJP *et al.* 1995) and images processed and analyzed as described elsewhere (LAGERWEIJ *et al.* 1994) with dedicated software (TMR 2000 2.0.27.16; Inspektor Research Systems, Amsterdam, The Netherlands). Three 500-µm scans were taken for each section and averaged. For each dentinal specimen the integrated mineral loss (IML, vol%×µm) data was obtained.

Multiplex ligation-dependent probe amplification (MLPA)

The multiplex ligation-dependent probe amplification (MLPA) method (SCHOUTEN *et al.* 2002) is a multiplex assay recently validated for identification and relative quantification of oral microbial species (TEREFEWORK *et al.* 2008). It allows identifying up to 50 unique sequences in a single reaction. The MLPA probe mix is

added to a denatured genomic DNA. For a positive analysis two parts of each probe need to hybridize to immediately adjacent target sequences. MLPA discriminates a single nucleotide difference. Only in the absence of a mismatch at the probe ligation site the two probe oligonucleotides will be ligated. The ligation products will then be amplified in a single PCR reaction.

DNA was extracted as described previously (ZAURA *et al.* 2009) using an AGOWA mag mini DNA isolation kit following the instructions of the manufacturer (AGOWA GmbH, Germany) and quantified using a Nanodrop (Nanodrop ND-1000; NanoDrop Technologies, Montchanin, DE, USA).

Per sample 5 ng DNA was used. A control plasmid (33 zmol) containing five control target sequences (Table 1) for hybridization efficiency control and signal intensity normalization was added to the sample DNA template. In total, 20 MLPA probes targeting different oral microorganisms (Table 1) were included in the panel. The probes were tested on DNA obtained from closely related species with a high degree of homology with the 16S rDNA sequences as described previously (TEREFEWORK *et al*. 2008).

The MLPA reaction was performed using a thermocycler (Tprofessional thermocycler, Biometra GmbH, Göttingen, Germany) as described previously (TEREFEWORK *et al.* 2008) and detailed at http://www.mrc-holland.com. MLPA reagents were provided by MRC-Holland (Amsterdam, the Netherlands). The PCR products were separated by capillary electrophoresis using a CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., California, USA). The relative amount of each probe was calculated as a proportion of the sum of all probes. Principal component analysis was used on log2 transformed data and calculated using PAST software (HAMMER *et al.* 2001).

Real time PCR

To quantify *S. mutans* and *L. rhamnosus* in the microcosms, primers/probes sets (Table 2) were designed based on the regions of identity within 16S rDNA of these two microorganisms. The probes are oligonucleotides with a reporter fluorescent dye (FAM) at 5′-ends and a quencher dye (BBQ) attached at 3′-ends. Previously

designed and evaluated universal primers and probe (NADKARNI *et al.* 2002) were used to quantify total microbial load.

Amplification and detection of DNA by RT-PCR was performed with the Lightcycler® 480 Real-Time PCR Instrument (Roche Diagnostics GmbH, Germany) using 96 well plates. The DNA samples were run in duplicate and the average of threshold cycle (Ct) values was calculated. The PCR reactions were carried out in a total of 10 µL or 20 µL containing PCR probe mix (2x), 5 ng/µL DNA templates, primers/probe and milliQ water. The PCR program for *S. mutans* and total 16S rDNA was 95 °C for 10 min, 60 cycles of 95 °C for 10 sec, 60 °C for 30 sec, and 72 °C for 30 sec. The program for *L. rhamnosus* was 95 °C for 10 min, 60 cycles of 95 °C for 10 sec, 58 °C for 30 sec, and 72 °C for 30 sec. Standard curves were generated from duplicate 10-fold dilution series of purified genomic DNA from three different microorganisms: *S. mutans* UA159, *L. rhamnosus* GG (ATCC 53103) and *E. coli*. The standard curve prepared with these dilutions was used in each assay. The proportion of CFU equivalent of 16S rDNA of *S. mutans* and *L. rhamnosus* over universal probe counts was calculated.

Statistical analyses

The Statistical package for the Social Sciences (SPSS version 18) was used to perform statistical analyses. For all tests on single and dual species experiments, triplicate samples from three independent experiments were averaged into a single value per growth condition. The growth of LGG and *S. mutans* in single species biofilms at 24 h and 48 h and the effects of LGG on the growth of *S. mutans* in the dual species biofilms were calculated using the Independent sample T-test on log transformed CFU counts and pH data.

In the microcosm experiments, triplicate samples from two independent experiments were averaged into a single value per individual saliva and growth condition. The effects of the probiotic LGG and sucrose on the pH, the IML, and *S. mutans* counts of the 72-h old microcosms were calculated using the Independent sample T-test. The proportion of CFU equivalent of 16S rDNA of *S. mutans* and *L. rhamnosus* per total bacterial 16S rDNA (universal probe counts) was calculated

and used in statistical analyses. Pearson's correlation analysis was used to correlate the outcome of two molecular methods in measuring relative abundance of LGG in microcosm samples – MLPA and real-time PCR, and to correlate the relative amount of viable cells of mutans streptococci per total viable counts with the relative amount of *S. mutans* 16S rDNA per total 16S rDNA. The significance level of all tests was 0.05.

Table 1. MLPA probe panel used in this study.

Size*	MLPA probe target**	Probe sequences***
202	*Actinomyces gerencseriae*	GTGCCGGTTGTCCTTCGGAGACGGAGGGTCCTCCCT**TTGCGGGGGTCGGTTCACAG** (1074 – 1131)
337	*Actinomyces naeslundii*	CGAACGGTGAAGGGGCCTGCTTTTGTGGGTCCTGGA**TGAGTGGCGAACGGGTGAGTAACACGTG** (74 – 159)
364	*Actinomyces odontolyticus*	CGGGTTGTGAACCTCTTTCGCTCATGGTCAAGCCGCA**ACTCAAGGTTGTGGTGAGGG** (485 – 546)
355	*Aggregatibacter actinomycetemcomitans*	TCGTTCTCGCCTTGTAAGCCGAC**TACTCTGCCGGCTGCACGAATTAACGCCA**
214	*Bifidobacterium dentium*	GCCGGTACAGCGGGATGCGACAT**GGCGACATGGAGCGGATCCCTGAAAACCGGTC** (1329 – 1383)
190	*Candida albicans*	CGAATCGCATGGCCTTGTGCTGGCGATGGTTCATTC**AAATTTCTGCCCTATCAACTTTC** (261 – 308)
283	*Capnocytophaga gingivalis*	GGGAAGAATAAGGTGTACGTGTACATTGA**TGACGGTACCATATGAATAAGCATCGGCTAAC** (504 – 573)
292	*Fusobacterium nucleatum*	GTGAGTAACGCGTAAAGAATTTGCCTCACAGCT**AGGGACAACATTTGGAAACGAATGCTAATAC** (147 – 210)
341	*Lactobacillus acidophilus*	GAGATTCGCTTGCCTTCGCAGGCTTGCTCCTCGTTGT**ACTGTCCATTGTAGCACGTGTGTAG** (1316 – 1368)
373	*Leptotrichia buccalis*	CGATGATTACTGGGTGTGGGCATGAA**GAGTGTCCGTGCCGAAGCTAATGCGATAAGTA** (888 – 945)
220	*Lactobacillus salivarius*	GAACACGAGTGAGAGTAACTGTTCATTCG**ATGACGGTATCTAACCAGCAAGTCACGGC** (499 – 571)
310	*Micromonas micros*	CAAAGATTTATCGGTGTAAGAAGGGCTCGCGTCTGA**TTAGCTAGTTGGAAGGGTAAAGGCCTACCAA** (247 – 331)
226	*Neisseria mucosa*	GAACTGCGTTCTGAACTGGGTGA**CTAGAGTGTGTCAGAGGGAGGTAGAATTCCAC** (693 – 749)
274	*Porphyromonas gingivalis*	GGTTGTTCGGTAAGTCAGCGGTGAAACCTGAG**CGCTCAACGTTCAGCCTGCCGTTGAAACTGCC** (649 – 713)
409	*Prevotella* common	CGTGCCAGCAGCCGCGGTAATACGGAA**GGTCCAGGCGTTATCCGGATTTATTGGGTTT** (579 – 636)
172	*Rothia dentocariosa*	CGAAGAACCTTACCAAGGCTTGACATATACTGGACTG**CGTCAGAGATGGCGTTTCCCTTCGGGGCTGGTATACAG** (1048 – 1131)
301	*Streptococcus mutans*	GCTATGGCTCAACCATAGTGTGCTCTGGAAACTGTCT**GACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTA** (675 – 753)
232	*Streptococcus sobrinus*	GGAAACGATAGCTAATACCGCATAAGAGGA**GTTAACTCATGTTAACTGTTTAAAAGAAGCCATTGCTTCA** (193 – 275)
252	*Veillonella parvula*	CCGTGATGGGATGGAAACTGCTGATCTAGAGTATCG**GAGAGGAAAGTGGAATTCCCATGT** (689 – 750)
196	*Lactobacillus rhamnosus*	GGCGCCTTATGTCCGGTTAATTACC**ACCGAAAAAGGCCCGAATGGCGATGAAA**
142	CP0101 (BCL2L2 gene)	CAGCAGTGGGGGCTGGGCGGA**GTTCACAGCTCTATACGGGGACGGGGCCCTG**
229	CP0103 (BIRC5 gene)	GAGAAAGAGCCAAGAACAAAATTGCAAAGG**AAACCAACAATAAGAAGAAAGAATTTGAGGAAACTGCGAAGAA**
463	CP0104 (BMF gene)	GCTTCATGTGCAGCAACACCAGCA**GAACCAAAATCGTGTGTGGTGGCAGATCCTCCTCTTC**
427	CP0107 (GZMB gene)	CGACATCATGCTACTGCAGCTGGAGA**GAAAGGCCAAGCGGACCAGAGCTGTGCAG**
229	CP0108 (BAK1 gene)	GCCTATGAGTACTTCACCAAGATTGCCA**CCAGCCTGTTTGAGAGTGGCATCAATTGGGGCC**

* - Length (nt) of the MLPA fragment

** - The upper panel indicates the probe targets for 20 oral microorganisms (16S rDNA in all but *A. actinomycetemcomitans* and *L. rhamnosus* where the target sequence was GRoEL and methionine synthase, respectively); the lower panel indicates the probe targets for control probes – human genes.

*** - Only hybridizing sequences included. Left probe oligonucleotides (plain), right probe oligonucleotides (**bold**). Numbers in brackets indicate nucleotide positions in *E. coli*.

Table 2. Primers and probes used in real-time PCR.

Name	Sequences (5′ - 3′)	References
S. mutans primer forward	GGGACGCAAGGAAACACACT	This study
S. mutans primer reverse	ATAGGCAGGTTACCTACGCGTTACTC	This study
S. mutans probe	6FAM-TGCTTGCACACCG+T+GTTT-BBQ	This study
L. rhamnosus primer forward	CAGAAATCAAAGAAGACAAACTCGTTAAG	This study
L. rhamnosus primer reverse	CCATGTAAACGGACAATGGGAGT	This study
L. rhamnosus probe	6FAM-CGGATTTCCAAAGCAATTCTTAACGATGAAAATG-BBQ	This study
Universal primer forward	TCCTACGGGAGGCAGCAGT	Nadkarni MA *et al*, 2002
Universal primer reverse	GGACTACCAGGGTATCTAATCCTGTT	Nadkarni MA *et al*, 2002
Universal probe	6FAM-CGTATTACCGCGGCTGCTGGCAC-TAMRA	Nadkarni MA *et al*, 2002

RESULTS

Dual and single species biofilms

First, the ability of *L. rhamnosus* GG (LGG) and *S. mutans* UA159 (SM) to grow in a biofilm were tested. Both strains were able to form a biofilm when incubated as a monoculture in the Active Attachment Biofilm model (Figure 1). The average LGG counts at 24 h (6.7 ± 0.6 log CFU) and 48 h (7.8 ± 0.3 log CFU) were significantly different ($p<0.001$), whereas SM had reached the maximum already after 24 h (Figure 1). LGG and SM formed a dual species biofilm irrespectively whether both strains were inoculated simultaneously or at different time points (T = 0 h and T = 24 h) (Figure 1).

Compared with single SM biofilm (SM 48; 7.5 ± 0.3 log CFU), the simultaneously inoculated biofilm of LGG and SM (T0 LGG + T0 SM; 6.9 ± 0.5 log CFU of SM) showed some, though a significant ($p=0.005$) inhibitory effect on SM growth. The existing LGG biofilm significantly ($p<0.001$) inhibited the growth of SM added at 24 h and grown for another 24 h (T0 LGG + T24 SM; 5.8 ± 0.4 log CFU of MS) compared to SM alone after 24 h of growth (SM 24; 8 ± 0.1 log CFU). However the existing SM biofilm was not affected by adding LGG at 24 h (T0 SM + T24 LGG; 7.7 ± 0.2 log CFU of SM) ($p=0.282$). The existing SM biofilm significantly enhanced the growth of LGG (T0 SM + T24 LGG; 7.6 ± 0.3 log CFU of LGG) compared to the LGG biofilm grown alone after 24 h (T0 LGG; 6.7 ± 0.6 log CFU) ($p=0.001$).

The pH of the spent medium in monoculture of each species did not significantly change with time and stayed around pH 7 in the LGG biofilm and decreased to pH 5 in the SM biofilm. The pH of the spent medium (Figure 1) decreased from pH 7 to pH 4.8 (SD 0.6) when SM was part of the original inoculum (T0 LGG + T0 SM and T0 SM + T24 LGG), and was significantly lower than the pH in the spent medium when SM was added to an existing LGG biofilm (T0 LGG + T24 SM; pH 6.6 ± 0.3) ($p<0.001$). Adding LGG to an existing MS biofilm (T0 SM + T24 LGG; pH 4.5 ± 0.3) did not lower the pH of the spent medium more than the pH of 48 h-old single species SM biofilms (SM 48 h; pH 4.9 ± 0.6), ($p> 0.05$).

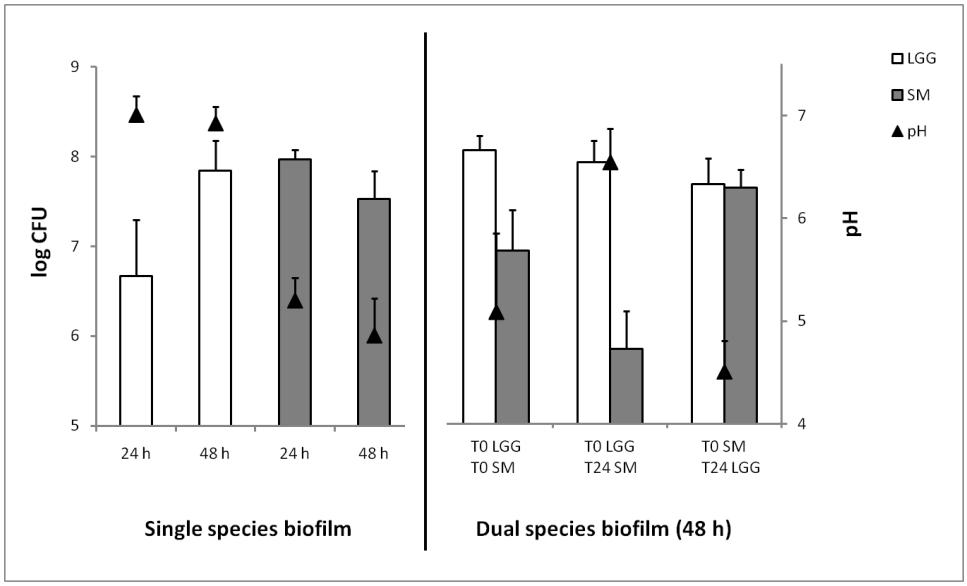

Figure 1. Average log-transformed CFUs of *Streptococcus mutans* (SM) and *Lactobacillus rhamnosus* GG (LGG) in single and dual species biofilms and the pH of the spent medium (secondary Y axis). Error bars indicate positive standard deviations obtained from triplicate samples of three independent experiments. Single species biofilms were harvested at 24 h and 48 h. Dual species biofilms were inoculated in three ways: 1) both LGG and SM simultaneously (T0 LGG, T0 SM), 2) LGG first, then SM after 24 h (T0 LGG, T24 SM), and 3) SM first, then LGG after 24 h (T0 SM, T24 LGG).

Microcosms derived from individual salivas

Ability of probiotic L. rhamnosus GG to establish into microcosms

We divided six individual salivas into two groups based on mutans streptococci (MS) counts in the saliva: 1) A high MS group comprising more than 10^5 cfu/mL mutans streptococci in saliva and 2) A low MS group with less than 10^5 cfu/mL of salivary MS (Table 3).

We assessed the ability of LGG to establish itself into microcosms. The MLPA and RT-PCR results showed that 16S rDNA of *L. rhamnosus* was present in all microcosms when added to the inoculum, irrespective of the individual saliva (Figure 2, Table 3). Sucrose-exposed microcosms harbored significantly more LGG than microcosms grown in the absence of sucrose ($p<0.001$). There was a significant

correlation between LGG proportion in the microcosms when assessed by either MLPA or RT-PCR (p= 0.002, Pearson correlation coefficient 0.494).

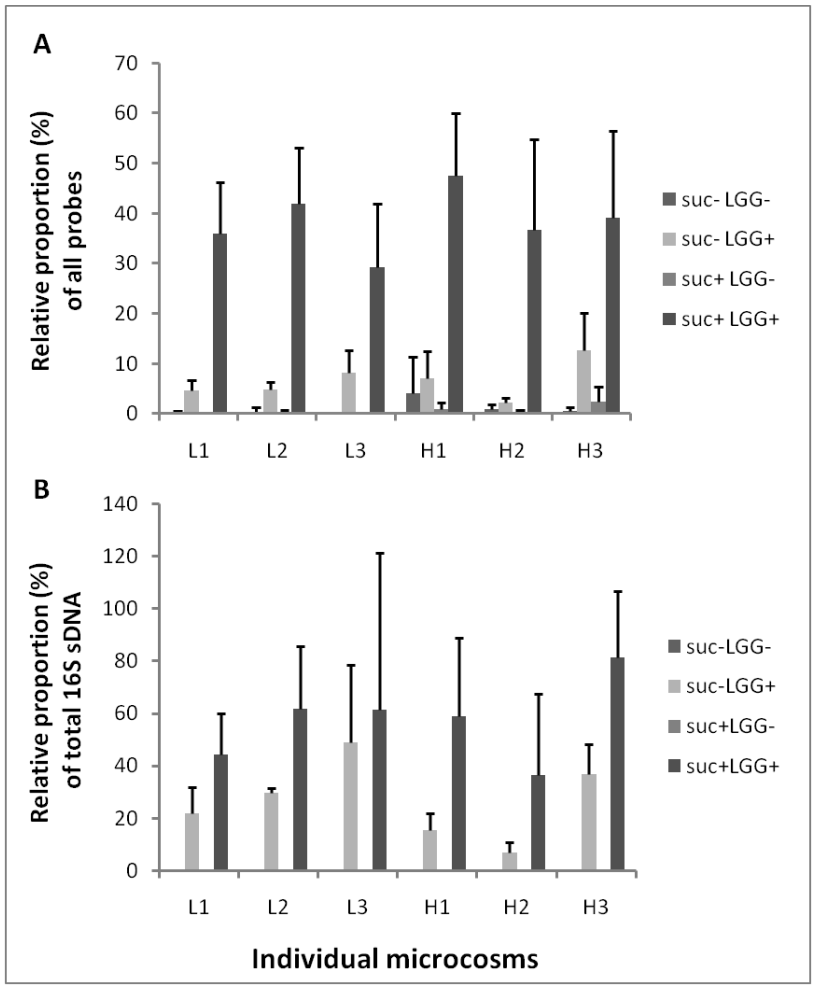

Figure 2. Relative abundance of LGG in microcosms at different growth conditions and per individual inoculum by: (**A**) MLPA – multiplex ligation-dependent probe amplification technique as a proportion of sum of all 20 microbial probes in the MLPA panel (Table 1) and (**B**) by real-time PCR of *L. rhamnosus* 16S rDNA as a proportion of total 16S rDNA. The growth conditions were either with or without sucrose in the medium (suc+/suc-) and with or without LGG (LGG+/LGG-). Six different inocula for the microcosms were divided in low mutans streptococci group (L samples) and in high mutans streptococci group (H samples) based on the viable mutans streptococci counts in saliva.

The impact of sucrose and LGG on the cariogenic potential of microcosms

Sucrose had a significant effect on pH, integrated mineral loss (IML) in dentin and lactate production by microcosms ($p<0.001$), irrespective of the saliva donor or the abundance of mutans streptococci in the inoculum (Figure 3, Table 3). The amount of mutans streptococci cells (log CFU) and the CFU equivalent of 16S rDNA of *S. mutans* increased significantly ($p<0.001$) in the presence of sucrose.

LGG had no significant effect on pH, IML and lactate production in the presence of sucrose ($p>0.05$) (Figure 3, Table 3). However, significantly less lactate was produced by microcosms grown without a sucrose supplementation in the presence of LGG than without LGG ($p<0.001$) (Table 3). Moreover, microcosms grown in the presence of LGG harbored less mutans streptococci ($p<0.05$) and had a lower relative abundance of the CFU equivalent of 16S rDNA of *S. mutans* ($p=0.013$) than the microcosms grown in the absence of LGG (Figure 3, Table 3).

There was a significant correlation between *S. mutans* proportion using CFU and RT-PCR ($p<0.001$; Pearson correlation coefficient 0.823). However, there was no correlation between MLPA and RT-PCR of *S. mutans* or between *S. mutans* proportion by MLPA and the proportion of mutans streptococci CFU counts.

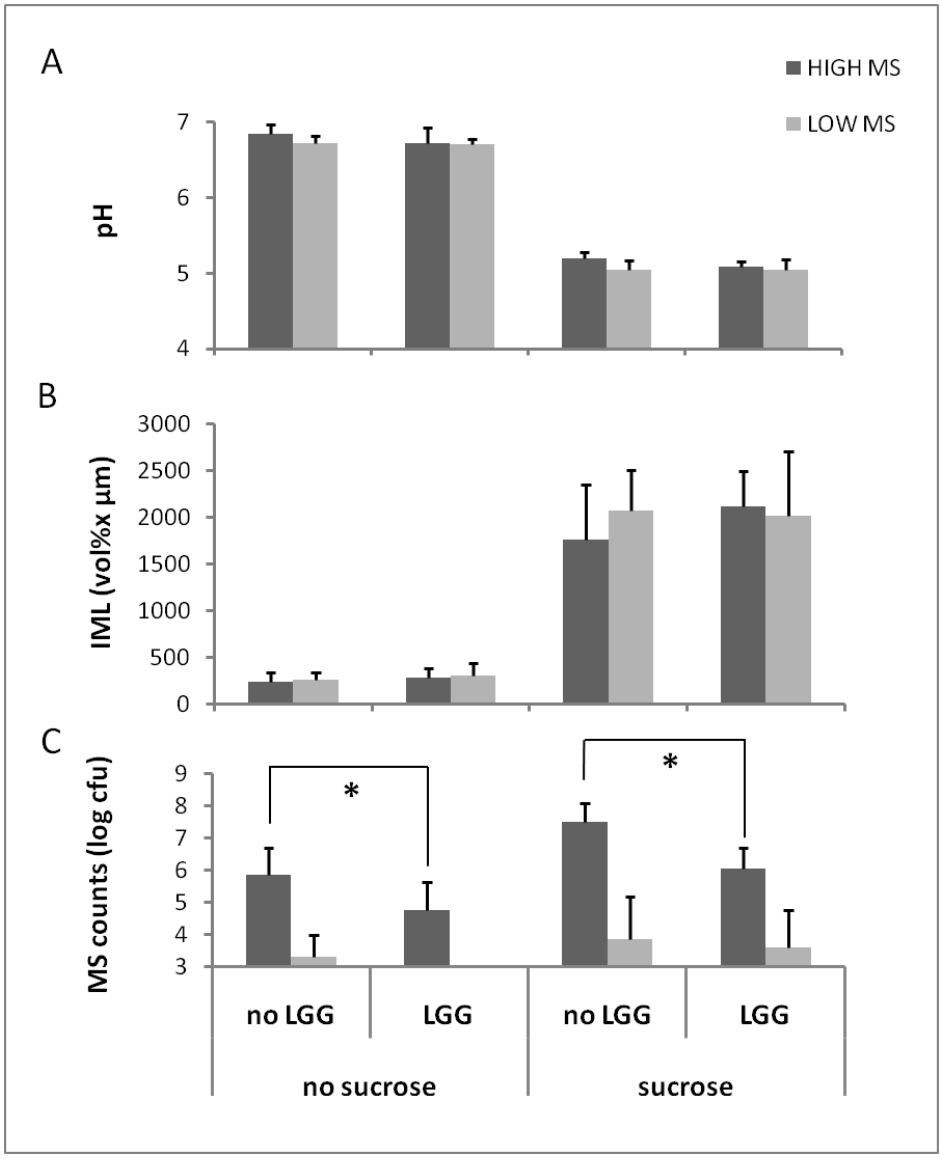

Figure 3. Effects of sucrose and LGG in microcosms originated from high mutans streptococci (high MS) inocula and low MS inocula on (**A**) average pH of the spent medium; (**B**) average integrated mineral loss (IML) in dentin; and (**C**) average log-transformed CFU counts of mutans streptococci (MS). Error bars indicate positive standard errors from triplicate samples of two independent experiments. Detection limit for MS was 1 x 10^3 CFU. The asterisk indicates statistically significant difference ($p<0.05$).

Table 3. Average real-time PCR logCFU equivalents of *S. mutans* and *L. rhamnosus*, the amount of lactate produced by biofilms and integrated mineral loss (IML) in dentin from microcosms grown with or without sucrose (suc+/suc-) and with or without probiotic strain *L. rhamnosus* LGG (LGG+/LGG-) per individual inoculum. The data were generated from triplicate samples at different experimental conditions of two independent experiments.

Output parameter	Growth condition	Low MS inoculum*			High MS inoculum*			Average all
		L1	L2	L3	H1	H2	H3	
RT-PCR *S. mutans* (logCFU equivalent (SD))	Inoculum	n.d**	4.1 (0.03)	n.d	6.5 (0.03)	5.4 (0)	5.0 (0.01)	5.2 (1.0)
	suc-/LGG-	n.d	3.7 (0.3)	n.d	4.4 (0.4)	3.4 (0.3)	3.6 (0.3)	3.5 (0.5)
	suc-/LGG+	n.d	3.3 (0.7)	n.d	3.9 (0.2)	3.3 (0.3)	3.3 (0.2)	3.3 (0.3)
	suc+/LGG-	n.d	3.9 (1.1)	3.8 (1.0)	6.5 (0.4)	5.1 (1.0)	4.7 (0.9)	4.5 (1.2)
	suc+/LGG+	n.d	4.6 (0.1)	3.1 (0.3)	5.7 (0.4)	4.6 (0.4)	4.0 (1.2)	4.2 (1.0)
RT-PCR *L. rhamnosus* (logCFU equivalent (SD))	Inoculum	4.3 (0.1)	4.2 (0)	4.2 (0.1)	4.3 (0.3)	4.3 (0.03)	4.3 (0.2)	4.3 (0.04)
	suc-/LGG-	4.4 (0.6)	4.3 (0.2)	4.2 (0.2)	4.3 (0.2)	4.2 (0.1)	4.3 (0.2)	4.3 (0.1)
	suc-/LGG+	7.0 (0.5)	7.0 (0.4)	7.3 (0.7)	6.8 (0.5)	6.6 (0.7)	7.7 (0.2)	7.1 (0.4)
	suc+/LGG-	4.2 (0.1)	4.3 (0.2)	4.3 (0.2)	4.4 (0.3)	4.2 (0)	4.3 (0.2)	4.3 (0.1)
	suc+/LGG+	7.7 (0.4)	7.9 (0.4)	7.6 (0.7)	7.9 (0.3)	7.8 (0.6)	7.9 (0.8)	7.8 (0.1)
Lactate (mM (SD))	suc-/LGG-	11.2 (2.7)	10.6 (0.6)	11.8 (0.9)	9.9 (1.5)	11 (1.7)	12.7 (0.3)	11.2 (1.7)
	suc-/LGG+	9.4 (2.2)	9.2 (0.7)	11 (0.5)	8 (1.4)	9.8 (0.8)	11.2 (0.7)	9.8 (1.5)
	suc+/LGG-	9.1 (1)	7.5 (2)	10 (1.4)	6.7 (1.2)	8.1 (0.5)	9.6 (0.5)	8.5 (1.6)
	suc+/LGG+	9.2 (1.2)	8.3 (2)	11.4 (1.1)	6.1 (0.9)	6.9 (0.5)	9.9 (0.9)	8.6 (2.1)
IML (vol%x μm (SD))	suc-/LGG-	249 (77)	267 (86)	279 (80)	206 (127)	262 (100)	249 (89)	252 (91)
	suc-/LGG+	288 (97)	339 (201)	296 (53)	273 (103)	269 (76)	327 (137)	298 (115)
	suc+/LGG-	2135 (325)	1976 (516)	2118 (511)	1666 (813)	1780 (270)	1857 (685)	1922 (536)
	suc+/LGG+	2052 (479)	1919 (948)	2103 (665)	2138 (485)	2083 (447)	2146 (277)	2073 (549)

* - saliva from 6 individuals was used as inoculum and the microcosm samples were dichotomized into two groups – low (L samples) and high (H samples) mutans streptococci containing inocula. The cutoff was 10^5 CFU of MS/mL saliva.

** - n.d., not detected, the detection limit of MS CFU and *S. mutans* RT-PCR probes equivalent to 10^3 cells/mL.

Effects of sucrose and LGG on the compositional stability of oral microbial communities

A panel of 20 MLPA probes targeting different oral microorganisms (Table 1) was used to study the microcosm composition under different growth conditions. Analysis on MLPA profiles revealed that all 20 probes resulted in a detectable signal (Table 4). On average, 10.5 (5 to 17) probes gave a positive signal per individual sample. Microcosms grown in the presence of sucrose showed significantly lower proportions of *Fusobacterium nucleatum, Leptotrichia buccalis, Micromonas micros* and genus *Prevotella* and higher proportions of *S. mutans* and *S. sobrinus* than the microcosms grown in the absence of sucrose ($p<0.05$). As expected, the proportion of *L. rhamnosus* significantly increased if LGG was added to the inoculum ($p<0.001$). The proportion of *Actinomyces odontolyticus* and *S. mutans* decreased significantly in the simultaneous presence of LGG and sucrose ($p<0.05$), while the proportion of genus *Prevotella* decreased in the presence of LGG, irrespective the presence or absence of sucrose ($p<0.05$).

Dimensional reduction of the MLPA profile data by Principal Component Analysis (PCA) explained 71% of the total variance among the microcosm samples by the first three components (Figure 4, Table 5). The most determining component (PC1: 43% of variance) discriminated the samples grown in the simultaneous presence of LGG and sucrose from the other samples. *L. rhamnosus*, genus *Prevotella*, *L. buccalis* and *Veillonella parvula* were major contributors to component 1 (Table 5). The second component (PC2: 19% of variance) discriminated the samples originating from the two groups of inocula - low versus high MS counts in the individual salivas that were used as the inoculum. *F. nucleatum*, genus *Prevotella* and *V. parvula* contributed the most to the second component (Table 5).

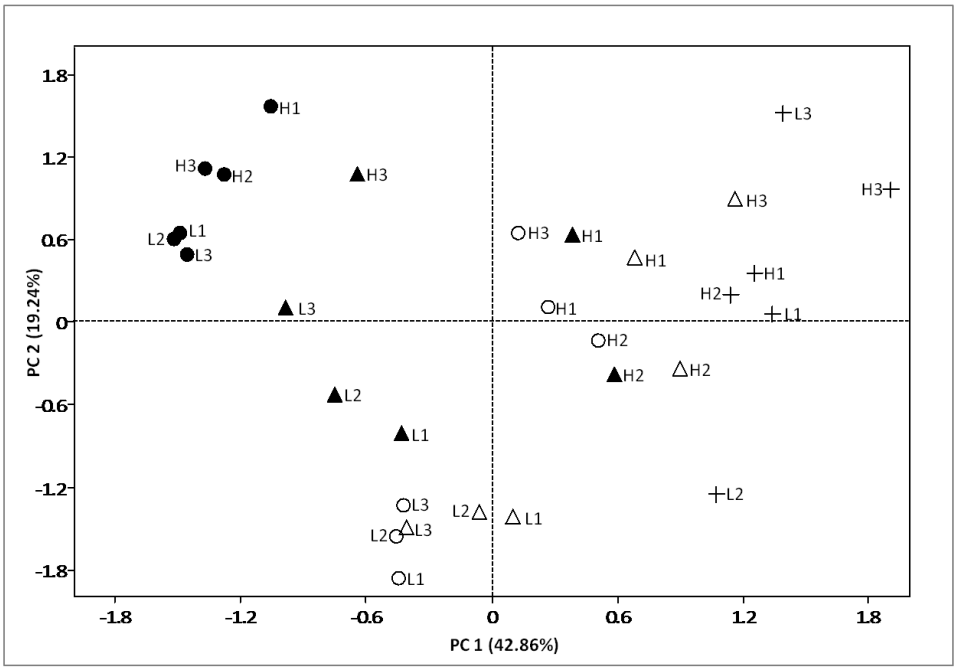

Figure 4. Principal Component Analysis (PCA) results of microcosm biofilms grown at different conditions from individual saliva donors. The PCA plot shows the first two principal components - PC1 (accounting for 42.86% of variance) and PC2 (19.24% of variance). MLPA microbial profile data were normalized as the proportion of sum of all probes and log2 tranformed. Cross (+) indicate six individual salivas used as inoculum; open triangles (Δ) – microcosm samples obtained at suc-/LGG- growth condition; filled triangles (▲) – samples grown at suc-/LGG+; open circles (○) – suc+/LGG-; filled circles (●) – suc+/LGG+. Symbols marked with L1, L2, L3 indicate low MS counts in the inoculum (individual saliva) of the microcosm; the symbols with H1, H2, H3 – high MS counts.

Table 4. Relative abundance of 20 microorganisms included in the MLPA probe panel in saliva-derived microcosms grown under four experimental growth conditions: with or without sucrose in the presence or absence of LGG.

MLPA probe target	Inoculum	Suc-/LGG-	Suc-/LGG+	Suc+/LGG-	Suc+/LGG+
A. gerencseriae	2.7 (5.9)	0.2 (0.4)	0.2 (0.6)	0.1 (0.2)	0.2 (0.4)
A. naeslundii	0.2 (0.3)	0.04 (0.2)	0.1 (0.1)	0	0
A. odontolyticus	18.3 (7.9)	31.2 (15.7)	38.1 (18.1)	33.3 (22.6)	15.1 (11.8)
A. actinomycetemcomitans	0	0.1 (0.3)	0	0.4 (1.3)	0.03 (0.2)
B. dentium	0	0.1 (0.3)	0.1 (0.4)	1.2 (2.9)	0.9 (2.2)
C. albicans	9.1 (7.3)	3.1 (2.1)	2.7 (2.1)	4.5 (4.3)	3.4 (2.6)
C. gingivalis	0.2 (0.5)	0	0	0	0
F. nucleatum	1.1 (1.0)	4.4 (5.2)	4.4 (5.7)	1.2 (3.7)	0.01 (0.04)
L. acidophilus	1.2 (2.8)	0.4 (0.6)	0.4 (1.2)	0.2 (0.3)	0.3 (0.8)
L. buccalis	6.0 (6.1)	0.3 (0.4)	0.4 (0.6)	0.02 (0.1)	0.01 (0.03)
L. rhamnosus	0.8 (1.1)	1.1 (3.1)	6.5 (5.0)	0.7 (1.4)	38.8 (14.0)
L. salivarius	0	0	0.06 (0.3)	0.1 (0.4)	0.02 (0.1)
N. mucosa	3.8 (4.8)	0.02 (0.1)	0.8 (0.3)	0.2 (0.4)	0.2 (0.4)
P. gingivalis	0	0	0.02 (0.1)	0	0.04 (0.2)
M. micros	0.2 (0.4)	1.4 (1.3)	0.9 (1.4)	0.2 (0.7)	0.02 (0.1)
Prevotella common	33.9 (13.6)	15.3 (17.4)	5.7 (9.8)	4.2 (6.3)	0.4 (1.0)
R. dentocariosa	1.5 (0.9)	0.03 (0.1)	0.004 (0.02)	0	0
S. mutans	0.4 (0.7)	0.04 (0.2)	0.03 (0.2)	0.7 (1.3)	0.02 (0.1)
S. sobrinus	1.1 (0.8)	0.2 (0.3)	0.3 (1.2)	1.0 (1.2)	0.4 (0.8)
V. parvula	19.6 (12.5)	42.1 (27.7)	40.1 (21.2)	52.1 (26.8)	40.1 (19.7)

Table 5. Loadings of the Principal Component Analysis of the MLPA microbial profile data. The five columns represent the five main principal components of the PCA. Values in the brackets indicate the variance explained by the respective component. Highlighted loadings are above the arbitrary significance threshold of 0.2 or -0.2. The positive bold values are highlighted 35% dark; the negative bold values are highlighted 15% dark.

MLPA probe target	PC 1 (42.9%)	PC 2 (19.2%)	PC 3 (14%)	PC 4 (8.8%)	PC 5 (4.3%)
A. actinomycetemcomitans	0.00545	0.03044	-0.04407	-0.04718	0.1397
A. gerencseriae	0.05982	-0.105	**0.2813**	**0.297**	**0.3761**
A. naeslundii	0.02592	0.01907	-0.00914	-0.00053	0.01105
A. odontolyticus	0.1138	0.1629	**-0.5201**	-0.1434	**0.2846**
B. dentium	-0.0381	0.08494	-0.0268	0.02155	**0.396**
C. albicans	0.08831	-0.07109	**0.4069**	-0.02058	-0.00462
C. gingivalis	0.01484	-0.02609	0.08221	0.05365	0.101
F. nucleatum	0.12	**-0.3876**	**-0.2576**	**0.6761**	-0.1789
L. acidophilus	0.01481	-0.0803	0.1884	**0.3188**	**0.3472**
L. buccalis	**0.2604**	0.1177	**0.2761**	-0.05438	**-0.5421**
L. rhamnosus	**-0.5158**	**0.6765**	0.06228	**0.4554**	-0.1033
L. salivarius	-0.00967	-0.00899	-0.01798	-0.02496	0.0232
N. mucosa	0.1586	0.02071	**0.4411**	0.03882	0.1072
P. gingivalis	-0.00609	0.000293	0.004237	0.01026	-0.01024
M. micros	0.04371	-0.1375	-0.1275	**0.2221**	-0.07142
Prevotella common	**0.7247**	**0.374**	-0.1464	**0.2094**	0.02238
R. dentocariosa	0.1169	-0.00767	0.1686	0.0494	0.006412
S. mutans	0.04097	0.00504	-0.00788	-0.0522	**0.2282**
S. sobrinus	0.02595	0.005421	0.1736	-0.1231	**0.2437**
V. parvula	**-0.2374**	**-0.4008**	-0.04513	0.01864	-0.06722

DISCUSSION

In this study we investigated the effects of a probiotic strain *L. rhamnosus* GG on *S. mutans* biofilms and on the cariogenic potential and the microbial composition of saliva-derived microcosms. Results from the dual species experiments revealed that LGG inhibited the growth of *S. mutans,* but did not affect the pH of the spent medium. LGG established into and inhibited the growth of mutans streptococci in complex saliva-derived biofilms as well, while it had no effect on the pH and the demineralization of dentin. Microbial community profiling by MLPA and subsequent PCA analyses revealed that simultaneous growth of the microcosms with LGG under heavy cariogenic conditions (sucrose-supplemented artificial saliva) introduced a compositional shift in the microbial community.

L. rhamnosus GG is a probiotic strain associated with health benefits. Tested for its ability to establish into the microcosms showed that LGG, like another probiotic *Lactobacillus salivarius* W24 (PHAM *et al.* 2009), was able to establish into saliva-derived communities irrespective of individual saliva donor. Although LGG does not ferment sucrose (SILVA *et al.* 1987), it became more abundant during growth with sucrose-supplemented medium than without sucrose. This suggests that low pH may modulate the growth and establishment of LGG into the microcosms. LGG may suppress directly the adherence/growth of other bacteria (HAUKIOJA *et al.* 2008a; MACK *et al.* 1999), it may compete for the space with harmful bacteria (ZAREIE *et al.* 2006) or, indirectly, may benefit from selection of acid-tolerant microorganisms to which both, lactobacilli and mutans streptococci belong (CHOU and WEIMER 1999; MCDONALD *et al.* 1990; SVENSATER *et* al. 1997; TAKAHASHI and YAMADA 1999).

We observed a significant reduction of mutans streptococci counts not only in dual species biofilms but also in complex oral microcosms that were grown in the presence of LGG and sucrose. So far no other *in vitro* study had used complex microbial communities to evaluate the effects of LGG. Our findings are in line with the clinical observations where mutans streptococci counts decreased due to regular exposures to LGG-containing products (AHOLA *et al.* 2002; NASE *et al.* 2001). Nevertheless, the addition of LGG to oral microcosms did not affect the pH and had no inhibitory effect on demineralization of dentin. However, it did reduce lactate

production by the microcosms grown in the absence of sucrose, suggesting that in the presence of sucrose other microorganisms besides mutans streptococci were responsible for high demineralizing potential of the microcosms.

For the microcosm experiments we selected six individual saliva donors categorized into two groups depending on mutans streptococci counts in saliva. However, once the salivas were exposed to the same highly cariogenic conditions (sucrose in the growth medium), they all resulted in microcosms producing similar outcomes – low pH and high level of demineralization of the underlying dentin, irrespective of the MS load in saliva that was used as inoculum. This again strengthens our assumption that other microorganisms besides mutans streptococci must have been involved in high cariogenicity of sucrose- and sucrose- plus probiotic-exposed biofilms. Previous studies have shown that 'low-pH' non mutans streptococci and other acidogenic bacteria are involved in cariogenic conditions and early caries development (LINGSTROM *et al.* 2000; SANSONE *et al.* 1993; TAKAHASHI and YAMADA 1999; VAN HOUTE *et al.* 1996; VAN HOUTE *et al.* 1991; VAN RUYVEN *et al.* 2000). High levels of non mutans streptococci in human dental plaques have been linked to an enhanced subject caries status (SANSONE *et al.* 1993; VAN HOUTE *et al.* 1991). Among the plaque samples with a high rate of pH reduction and a low minimum pH were samples with low and high levels of mutans streptococci (VAN HOUTE *et al.* 1991). 'White spot' lesion-associated plaques were shown to harbour a low level of mutans streptococci (VAN RUYVEN *et al.* 2000). Many oral microorganisms are capable of acidogenesis at a low pH (COTTER and HILL 2003) such as *Streptococcus oralis* (ALAM *et al.* 2000; TAKAHASHI and YAMADA 1999), *Streptococcus sanguis*, *Streptococcus gordonii*, *Streptococcus mitis* (TAKAHASHI and YAMADA 1999), *Actinomyces* and Bifidobacteria (VAN HOUTE *et al.* 1996).

In a previous study we demonstrated that denaturing gradient gel electrophoresis (DGGE) can be used to detect a microbial shift due to perturbation by a probiotic bacterium (PHAM *et al.* 2009). Here we report that a targeted approach - multiplex ligation-dependent probe amplification - MLPA can also reveal the changes in microcosm composition due to different inocula, environmental factors and perturbation with a foreign bacterium such as LGG. We observed a decrease of microorganisms that are not acid-tolerant such as *F. nucleatum, L. buccalis, M. micros*

and genus *Prevotella*, while, as expected, the proportions of acidogenic and aciduric *S. mutans* and *S. sobrinus* increased in the presence of sucrose in the growth medium. Furthermore, the presence of LGG and sucrose induced prominent compositional shifts in the microcosms: proportions of *S. mutans*, *A. odontolyticus* and genus *Prevotella* decreased under this growth condition. Genus *Prevotella* was the only one of the MLPA panel targets that was affected by LGG also in the absence of sucrose. Recently in a simple agar diffusion assay it was demonstrated that different clinical isolates of lactobacilli, including *L. rhamnosus*, possess antimicrobial activity against *Prevotella intermedia* (KOLL *et al.* 2008). Our results show that antagonistic interaction between *Lactobacillus* and *Prevotella* may occur even within complex polymicrobial biofilms.

The establishment of LGG into the saliva-derived microcosms was detected by using both MLPA and real-time PCR probes. There was a strong correlation in the proportion of LGG in microcosms using MLPA and real time PCR technique. Among the samples where LGG was not added, in few cases MLPA detected *L. rhamnosus* while RT-PCR did not. This is probably due to a relatively high detection limit - 10^4 cfu/mL of the RT-PCR probe for *L. rhamnosus*.

LGG reduced *S. mutans* counts in both, dual species biofilms and in microcosms, as it was observed by CFU data, MLPA and RT-PCR. There was a strong correlation in proportion of *S. mutans* using two of these techniques - viable counts by culturing and RT-PCR. However, there was no significant correlation between *S. mutans* load by MLPA and RT-PCR. The detection limit of *S. mutans* 16S rDNA in the sample by MLPA probe was too high (approximately 10^8 cells/mL) compared to RT-PCR probe (10^3 cells/mL). The MLPA probe of *S. mutans* worked well in the panel of nine MLPA probes (TEREFEWORK *et al.* 2008). However, when the panel was expanded to 20 probes, the sensitivity of the *S. mutans* probe in the MLPA reaction decreased. For further applicability of MLPA on oral microorganisms a more sensitive *S. mutans* probe, potentially based on another than the 16S gene sequence, is required.

The current *in vitro* model included prolonged episodes of low pH due to sucrose fermentation in between 8- and 16-hourly refreshment of the medium and is thus an extreme situation, unlikely to prevail in the oral cavity. This severe situation

allowed us to assess the cariogenic potential of the probiotic strain tested and to conclude that LGG, unlike the previously assessed probiotic *L. salivarius* W24 (PHAM *et al.* 2009), is not cariogenic under these extreme conditions. However, a potential anticariogenic effect of the probiotic may have been masked by the severity of the cariogenic situation and could therefore not be ruled out. Additional studies with brief exposures to fermentable carbohydrates should be performed to elucidate any inhibitory effect of LGG on cariogenic potential of oral microcosms.

In conclusion, we showed that the intestinal probiotic *L. rhamnosus* GG was not only able to establish into saliva-derived microcosms, but also reduced *S. mutans* counts in the microcosms and affected the compositional stability of the microbial communities.

ACKNOWLEDGEMENTS

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Chapter V - Pyrosequencing analysis of human saliva and saliva-derived microcosms

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*).

ABSTRACT

Microcosms are complex communities derived from natural source such as saliva, and are used to study microbial ecological shifts in controlled *in vitro* environments. The aim of the current study was to assess the effects of individual inocula on microbiome profiles of the microcosms derived from these individual inocula. Additionally, we aimed to compare the open-ended approach in microbiome profiling by 454 pyrosequencing with the targeted approach by multiplex ligation-dependent probe amplification (MLPA) used for microcosm characterization in our previous study (PHAM *et al*. 2011). To these ends, we amplified hypervariable region V5-V7 of 16S rDNA of individual microcosms and their respective inocula (human saliva screened for high and low counts of mutans streptococci (MS)). The microcosms were obtained under four growth conditions: plain medium, plain medium supplemented with a probiotic strain *Lactobacillus rhamnosus* GG (LGG), sucrose-supplemented medium and medium supplemented with sucrose and LGG (PHAM *et al*. 2011). Individually tagged amplicon libraries were pooled and sequenced by the GS FLX Titanium system of Roche 454 pyrosequencing technology. Subsequently, the data was processed using the RDP pipeline at Straightforward Novel Webinterface for Microbiome Analysis. In total, 386,869 high quality reads (average length 370 nt) were obtained and clustered in 923 Operational Taxonomic Units (OTUs) at 6% difference level. Microbiome profiles of the six inocula were clearly dichotomized in high and low MS-saliva groups. Two OTUs, classified as *Neisseria* and *Porphyromonas*, were significantly more abundant in the low MS-inocula, while two *Prevotella* OTUs were present in significantly higher proportion in the high MS-salivas. Microcosms differed significantly from the inocula. The representatives of phylum Firmicutes dominated the microcosms. The microbiome profiles of the microcosms clustered according to the type of the inoculum (low or high MS saliva). Both profiling methods, MLPA and pyrosequencing, showed comparable results in discriminating the effects of inocula and growth conditions. We conclude that the choice of the inoculum based on predefined criteria allows modeling of ecological shifts in saliva-derived microcosms. Comparison of targeted profiling by MLPA with high throughput sequencing by 454 pyrosequencing showed that the MLPA is a valid method in

microcosm profiling. The output of the pyrosequencing study will allow further improvement of the MLPA approach (and other targeted methods) by justifying the choice of sequences for specific probe panels.

INTRODUCTION

The oral cavity is a dynamic ecological habitat where an interplay between environmental and host factors determines the composition and function of the microbial communities. Health to disease equilibrium is determined by this interplay, a phenomenon which was introduced to oral microbiology research by Marsh as 'ecological plaque hypothesis' (MARSH 1994). According to this hypothesis, repeated episodes of low pH lead to suppression of acid-sensitive species and the outgrowth of microorganisms with an aciduric physiology. Together this leads to a shift towards the disease, dental caries.

Clinical studies provide the only realistic systems to study ecological shifts and their consequences. However, control of environmental parameters *in vivo* is cumbersome, if possible at all. *In vitro* model systems allow the control of most, if not all, environmental parameters, but have serious limitations. They either lack the complexity of natural communities, such as by focusing on biofilm consortia of few defined species (GUGGENHEIM *et al.* 2001; SHU *et al.* 2000), or are too complex to characterize their composition and to assess the impact of environmental factors, such as in plaque- or saliva-derived communities, called microcosms (MCBAIN *et al.* 2003; PRATTEN *et al.* 1998b; SISSONS 1997).

In order to characterize microcosm communities, numerous methods ranging from targeted profiling with selected probes, *e.g.*, by DNA-DNA checkerboard (FILOCHE *et al.* 2007b; SISSONS 1997) and multiplex ligation-dependent probe amplification (MLPA) (PHAM *et al.* 2011; TEREFEWORK *et al.* 2008) to open-ended fingerprinting by denaturing gradient gel electrophoresis (DGGE) (LEDDER *et al.* 2006; PHAM *et al.* 2009) have been applied. None of these methods, however, offers both quantifiable and untargeted (open-end) high resolution assessment of microcosm composition. Current developments in sequencing technologies allow the identification by high throughput parallel sequencing (454 pyrosequencing) of short hypervariable

fragments of small subunit ribosomal DNA of microorganisms at a high resolution (VOELKERDING *et al.* 2009). Due to relatively low bias and reducing costs, this method is gaining popularity and has been used in several clinical, oral microbiome studies (KEIJSER *et al.* 2008; LAZAREVIC *et al.* 2010; LI *et al.* 2010; ZAURA *et al.* 2009). So far, 454 pyrosequencing has been used to confirm the microbial diversity observed by DGGE profiling of a single microcosm sample, derived from saliva pooled of several individuals (TIAN *et al.* 2010).

In a previous study (PHAM *et al.* 2011), we obtained microcosms that differed in their inoculum source and in growth conditions. The expected differences in the community profiles were confirmed by the community analyses of targeting 20 selected microorganisms using the MLPA technique. The aim of the current study was to assess the effects of individual inocula on microbiome profiles of microcosms described in Pham *et al.* (2011) by 454 pyrosequencing and compare with the output of the MLPA profiling.

MATERIALS AND METHODS

In this study we pyrosequenced the hypervariable regions V5-V7 of 16S rDNA from the samples obtained and described in detail in our previous study (PHAM *et al.* 2011) in Chapter IV. The following is a summary of the experimental procedures and sample processing, followed by a description of DNA amplicon library preparation, pyrosequencing and data analysis.

Generation of microcosm biofilms

Saliva-derived microcosms were grown on bovine enamel-dentin discs using an Active Attachment Biofilm (AAB) model (EXTERKATE *et al.* 2010). The model consists of a custom-made stainless steel lid with nylon clamps that can accommodate 24 substrata and fit into a 24-well plate. The enamel-dentin discs were prepared and inserted into the nylon clamp in the lid of the AAB model.

The inoculum (human stimulated saliva) was prepared as described previously (PHAM *et al.* 2009). The use of human saliva was approved by institutional Review Board. We selected six individual salivas based on mutans streptococci (MS) counts

in saliva: 1) three salivas with more than 10^5 cfu/mL MS in saliva (High MS group) and 2) three salivas with less than 10^5 cfu/mL of salivary MS (Low MS group). The growth medium for microcosms comprised artificial saliva (McBain *et al.* 2005). The microcosms were grown individually at four experimental conditions: a) in plain medium (Suc-LGG-); b) in plain medium with *Lactobacillus rhamnosus* GG (LGG) (Suc-LGG+); c) in sucrose-supplemented medium (Suc+LGG-); d) in sucrose-supplemented medium with LGG (Suc+LGG+). The microcosms were harvested after 72 h. In conditions (b) and (d), 10^6 cfu/mL of LGG was added to the medium at the time of the inoculation. Medium was refreshed at 8, 24, 32, 48 and 56 h. Each of the six individual saliva-derived microcosm experiments was performed with triplicate samples and repeated twice.

Biofilm processing

After 72 h, the pH of the spent medium was measured and the acidogenicity test was performed: the biofilms were incubated with 0.2% glucose anaerobically for 3 h at 37 °C. After the incubation, the amount of L-lactic acid in the incubation fluid was determined enzymatically using a colorimetric assay (Gutmann and Wahlefeld 1974). After the acid test, the specimens with adherent biofilms were sonicated in PBS and then the biofilm samples were centrifuged. The pellets were stored at - 80 °C until DNA extraction and quantification (Zaura *et al.* 2009).

Assessment of mineral loss

The dentin/enamel specimens were used to assess the mineral content of the dentin by transversal microradiography (Lagerweij *et al.* 1994). Two 200-µm-thick sections were cut from the center of each cylinder perpendicularly to the surface and radiographed (Van Strijp *et al.* 1995), the images were processed and the integrated mineral loss (IML, vol%×µm) calculated.

DNA library preparation and pyrosequencing

For microbiome analyses, a maximum of 102 samples could be individually tagged with sample identification keys and sequenced. Of the 150 samples (6 inocula and

36 samples per each of the four growth conditions), we included all inocula (N=6), and at least two replicates of each of the individual microcosms per growth condition (Suc-LGG-: N=26; Suc-LGG+: N=24; Suc+LGG-: N=22; Suc+LGG+: N=24) for pyrosequencing.

PCR amplicon libraries of the small subunit ribosomal RNA gene V5-V7 hypervariable region were generated for the individual samples. PCR was performed using the forward primer 785F (GGATTAGATACCCBRGTAGTC) and the reverse primer 1175R (ACGTCRTCCCCDCCTTCCTC). The primers included the 454 Life Sciences (Branford, CT, USA) Adapter A (for forward primers) and B (for reverse primers) fused to the 5′ end of the 16S rDNA bacterial primer sequence and a unique 10 nt sample identification key.

The amplification mix contained 2 units of Pfu Ultra II Fusion HS DNA polymerase (Stratagene, CA, USA), 1 unit Buffer Pfu Ultra II [10x], including 2.0 mM $MgCl_2$ (Stratagene), 240 μM dNTP PurePeak DNA polymerase Mix (Pierce Nucleic Acid Technologies, Milwaukee, WI), 0.5 μM of each primer. After denaturation (95 °C; 2 min), 30 cycles were performed consisting of denaturation (94 °C; 30 sec), annealing (50 °C; 40 sec), and extension (72 °C; 80 sec). The amplicons were purified by means of the MinElute kit (Qiagen, Hilden, Germany). The quality and the size of the amplicons were analyzed on the Agilent 2100 Bioanalyser with the DNA 1000 Chip kit (Agilent Technologies, Santa Clara, CA, USA). The amplicon libraries were pooled in equimolar amounts and sequenced unidirectionally in the reverse direction (B-adaptor) by means of the Genome Sequencer FLX Titanium system (Roche, Basel, Switzerland).

Pyrosequencing data analysis

The sequences were processed using RDP pipeline (COLE *et al.* 2005) at SNoWMAn - Straightforward Novel Webinterface for Microbiome Analysis (Graz University of Technology, Bioinformatics https://epona.genome.tugraz.at/snowman/). Fasta files with sample sequences, sample identification key files, primer sequences and quality files were uploaded to the webinterface. Two mismatches in each primer sequence were allowed. In RDP preprocessing, the sequences were sorted by RDP Tagsorter

and trimmed by RDP Trimmer by removing primer sequences and low-quality data, sequences that do not have an exact match to the reverse primer, that have an ambiguous base call (N) in the sequence, or that are shorter than 150 nt after trimming. For sequence alignment, the bacteria_2008_12_24_ncbi16S_508_mod5 infernal alignment model was used. NaiveBayesian_rRNA_Classifier_2010_02_01_2.1 model was used for sequence classification. Sequences were clustered in operational taxonomic units (OTUs) within 6% difference level. Taxonomy assignment was accepted only if the assignment probability was 0.8 or higher.

Principal component analysis (PCA), calculation of the Shannon diversity index and the species dominance index were performed in PAST (HAMMER *et al.* 2001). PCA is a commonly used method to reduce the dimensions of multivariate data. Principal components are combined variables that explain a large part of the overall variance. Two-dimensional graphs of the principal components visualize clustering of the individual samples in the space determined by these components. The coefficients of the PCA loadings of each variable (OTU in this case) were used to estimate the impact of each individual OTU on the clustering of the samples in PCA. PCA was performed on normalized and log2 transformed OTU data. For the diversity index $(H' = -\Sigma p_i \ln p_i)$ the proportion of species *i* relative to the total number of species (p_i) is calculated, and then multiplied by the natural logarithm of this proportion ($\ln p_i$). The resulting product is summed across species, and multiplied by -1. This index accounts for both the abundance and the evenness of the species present. Ecological dominance is the degree to which a particular species is more numerous than its competitors in an ecological community, or makes up more of the biomass. It ranges from 0 (all taxa are equally present) to 1 (one taxon dominates the community completely). The dominance index is calculated as follows: $D = \Sigma (n_i/n)^2$ where n_i is the number of individuals of taxon *i* and *n* is total number of individuals.

To compare the diversity data (number of taxa, diversity index and dominance data) of the microcosms grown at four different conditions with the diversity data of the inoculum, we performed general linear model repeated measures (GLM-RM) test (SPSS, version 18). Independent samples T-test was used to compare the abundance of those OTUs, which had the highest coefficients of the PCA loadings, between the two different groups (SPSS, version 18). The significance level of all tests was 0.05.

To compare the pyrosequencing results with the MLPA profiles of the same samples (PHAM *et al*. 2011), the highest taxon (genus or higher taxonomic level) dataset was used. The data of the replicate samples were averaged into a single microbial profile per experiment (N=6 individual microcosm experiments), normalized and log2 transformed before being analyzed by PCA. The relation between the relative abundance of the MLPA probe for genus *Prevotella* and the final taxon *Prevotella* in the pyrosequencing dataset was assessed with nonparametric Spearman's rho correlation (the data was not normally distributed: Kolmogorov-Smirnov normality test $p<0.001$).

Supplementary files with full list of higher taxa (Supplementary file 1), OTUs at 6% difference (Supplementary file 2) and PCA results (Supplementary file 3) are accessible electronically at:

http://www.onderwijs.acta.nl/data/Supplementary_file_1_taxa_level_CP.zip

http://www.onderwijs.acta.nl/data/Supplementary_file_2_all_OTUs_CP.zip

http://www.onderwijs.acta.nl/data/Supplementary_file_3_PCA_results_without_OTU_94_CP.zip

RESULTS

Overall sequencing and taxonomy output

Sequencing resulted in 456,903 reads that passed the Roche 454 quality control algorithm. Of these, 450,815 reads passed trimming and filtering of the SNoWMAn RDP pipeline. In total, 389,932 reads with average length of 370 nt (355 – 378 nt) passed the orientation check and were used in merging, uniquing and alignment steps of the pipeline. This processing pipeline resulted in 126,347 unique sequences that were further used for clustering in operational taxonomic units (OTUs) at 99%, 97% and 94% similarity. Only the data from 94% similarity (6% OTUs) are described further. We have chosen 6% difference level as this has been accepted previously as a more conservative estimate of species definition than the 3% level (KEIJSER *et al.* 2008). Clustering at 6% level resulted in 1,841 OTUs. To further reduce the impact of sequencing errors or potential contaminants, a cut-off of at least 5 reads per OTU was applied to the data. Of all OTUs, 923 OTUs with 386,869 reads passed this cut-off. An individual sample harbored on average 3,863 reads (range 1,177 – 8,261

reads; SD 1,490). Before the microbial community analyses, the data were normalized to achieve equal number of reads per individual sample.

The 923 OTUs with 386,869 reads were classified into 64 higher taxa (genus or more inclusive taxon) belonging to seven phyla of the domain Bacteria (Figure 1, supplementary file 1). Firmicutes dominated the data set (92.2% of all reads), while 2.2% of the reads could not be classified to any of the known phyla at the probability of 0.8 or higher (Unclassified bacteria), 2% of reads were classified as Actinobacteria, 1.5% - as Bacteroidetes, 1.2% - as Fusobacteria, 0.9% - as Proteobacteria, 0.1% - as candidate division TM7 and only 9 reads (0.002%) – as candidate division SR1.

Taxonomical classification and ecological diversity per sample type

Among the six saliva samples used as inocula for the microcosms, 57% (SD 16.5) reads/sample were classified as Firmicutes, 19.9% (SD 7) - as Bacteroidetes, 10.1% (SD 2.7) – as Actinobacteria, 8.7% (SD 9.1) – as Proteobacteria, 2.3% (SD 1.7) – as Fusobacteria, 0.5% (SD 0.6) – as candidate division TM7, while 1.4% (SD 0.4) of reads per saliva sample could not be classified to any known phylum (Figure 2). Reads classified as candidate division SR1 were found in two out of six inocula (0.1%, SD 0.1). High MS inocula contained statistically significantly lower proportion of phylum Proteobacteria than low MS inocula ($p<0.05$).

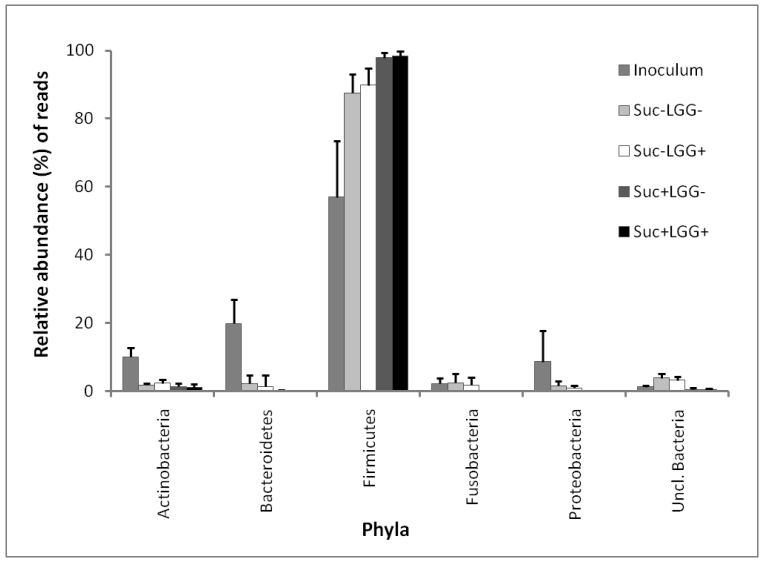

Figure 1. Average relative abundance (%) of reads classified to the five predominant phyla and unclassifiable bacteria in inoculum (N=6) and in microcosm samples grown at four conditions: in plain medium (Suc-LGG-) (N=26), in plain medium with probiotics (Suc-LGG+) (N=24), in sucrose-supplemented medium (Suc+LGG-) (N=22) and in sucrose-supplemented medium with probiotics (Suc+LGG+) (N=24). Error bars – standard deviations.

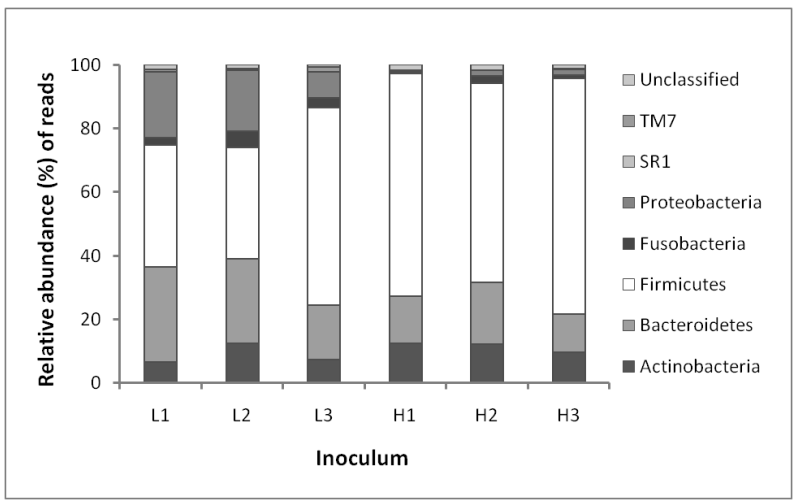

Figure 2. Average relative abundance (%) of the reads classified into the predominant microbial phyla by individual saliva that was used as the inoculum for microcosm experiments. L1, L2, L3 indicate the inocula with low mutans streptococci (MS) counts in the saliva; H1, H2, H3 –high MS inocula (MS >10^5 cfu/mL saliva).

On average, each inoculum contained 123 OTUs (SD 14) or taxa (Figure 3A). The microcosms grown with sucrose supplementation (113 OTUs (SD 30) and 111 OTUs (SD 35) without and with LGG, respectively) harbored similar number of OTUs compared to the inocula, while nearly double amounts of taxa ($p<0.05$) were found in the samples grown in the absence of sucrose – 208 (SD 43) and 212 (SD 33) OTUs without and with LGG, respectively. The 272 OTUs that were present only in the plain medium (Suc-) groups, were classified as Actinobacteria (8 OTUs), Bacteroidetes (24 OTUs), Firmicutes (132 OTUs), Fusobacteria (2 OTUs), Proteobacteria (22 OTUs) or remained unclassified (84 OTUs). Although each of these 272 OTUs were at a low abundance individually, together they accounted for 2.9% (SD 1.6) of the reads of the respective sample.

Representatives of genus *Veillonella* and *Streptococcus* dominated both the inocula and microcosms (Table 1, supplementary file 2). Four OTUs (#8 and #12 – *Streptococcus*; #7 – *Veillonella*; #18 – Unclassified Firmicutes) were found in all microcosm samples and in all inocula. Additionally, three OTUs (#9 - Lactobacillales; #44 – *Veillonella*; #70 – *Actinomyces*) were found in at least 80% of the samples. Among the six inocula, 26 OTUs were found in all, and 22 OTUs – in all but one inoculum. The predominant and 100%-prevalent inoculum-taxa (OTUs) were classified to genus *Veillonella* (#7), *Streptococcus* (#8, #12), *Actinomyces* (#70), *Prevotella* (#137, #201), *Rothia* (#555), *Porphyromonas* (#336), *Neisseria* (#662), *Gemella* (#1146) and order Lactobacillales (#9).

Apart from the decrease in the number of taxa (OTUs), growth in the presence of sucrose resulted in statistically significantly increased ecological dominance (Figure 3B) and in decreased ecological diversity (Figure 3C) of the community compared to those in the other groups.

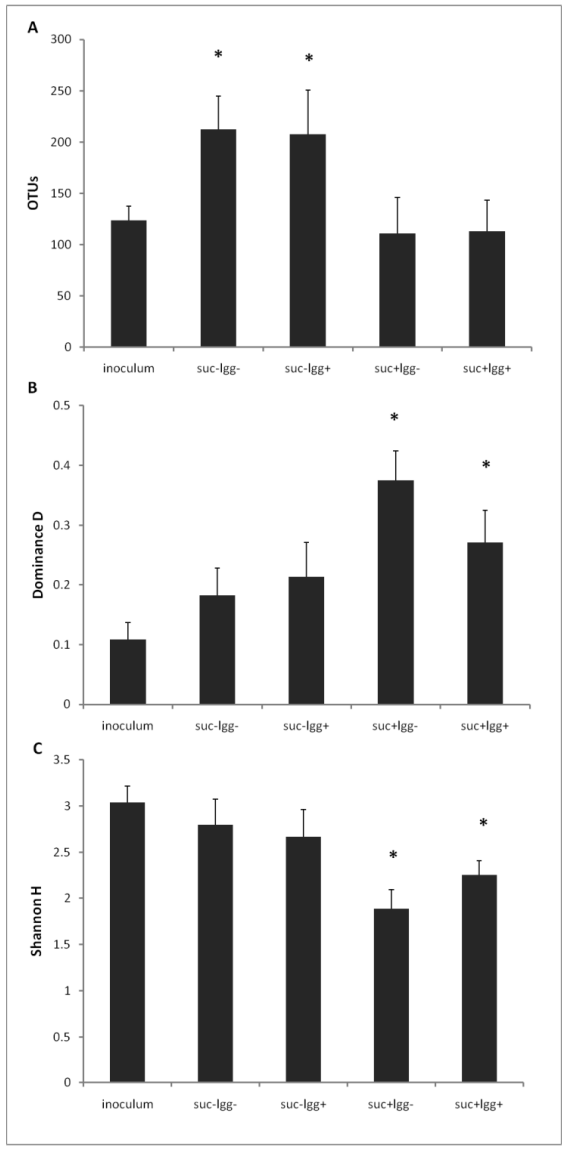

Figure 3. Output of the diversity statistics by sample type (inoculum and microcosms obtained from four different growth conditions: plain medium (Suc-LGG-), plain medium with LGG (Suc-LGG+), sucrose-supplemented medium (Suc+LGG-) and sucrose-supplemented medium with LGG (Suc+LGG+)): (**A**) species richness (number of OTUs or taxa); (**B**) Dominance index; (**C**) Shannon H diversity index. The data are average values from replicates of two independent experiments per inoculum. Error bars – standard deviations. * - statistically significantly different from the groups without the asterisk ($p<0.05$; GLM-RM test).

Table 1. Relative abundance (%) of top 30 OTUs per sample group. Data are average (standard deviation) values from samples belonging to five groups: inoculum (N=6), microcosms grown in the plain medium (Suc-LGG-) (N=26), in the plain medium with probiotics (Suc-LGG+) (N=24), in sucrose-supplemented medium (Suc+LGG-) (N=22) and in sucrose-supplemented medium with probiotics (Suc+LGG+) (N=24). Full list of OTUs per individual sample is available in the supplementary file 2.

OTU	Final Taxon	Inoculum	Suc-LGG-	Suc-LGG+	Suc+LGG-	Suc+LGG+
7	*Veillonella*	17.7 (10.8)	31.6 (13.8)	37.9 (12.3)	55.1 (13.8)	46.3 (13.5)
12	*Streptococcus*	10.4 (5.2)	19.6 (5.3)	17.3 (7.4)	6.5 (17.3)	1.3 (1.2)
8	*Streptococcus*	5.4 (2.7)	7.8 (8.2)	8.6 (7.6)	6.8 (2.7)	5.2 (3.5)
9	Lactobacillales	10.5 (11.0)	0.5 (0.5)	0.3 (0.3)	7.5 (3.9)	5.8 (3.9)
94	*Lactobacillaceae*	0	0.003 (0.01)	2.2 (1.5)	0.01 (0.01)	14.7 (6.5)
15	*Streptococcaceae*	1.5 (3.1)	0.9 (1.7)	0.7 (1.2)	4.0 (4.7)	3.5 (4.5)
70	*Actinomyces*	4.3 (1.9)	1.5 (0.6)	2.1 (1.0)	0.9 (0.6)	0.6 (0.4)
46	*Streptococcus*	0.3 (0.2)	3.9 (3.8)	2.5 (2.0)	0.5 (0.4)	0.3 (0.4)
662	*Neisseria*	7.0 (7.8)	0.1 (0.2)	0.1 (0.1)	0	0.001 (0.004)
18	Firmicutes	0.3 (0.2)	2.3 (0.7)	2.4 (0.6)	1.5 (0.7)	0.7 (0.4)
336	*Porphyromonas*	6.3 (7.3)	0.2 (0.7)	0.03 (0.07)	0.001 (0.003)	0
95	*Veillonella*	0.2 (0.2)	0.5 (0.5)	0.7 (0.4)	2.5 (0.8)	1.5 (0.5)
42	*Fusobacterium*	1.5 (1.2)	2.3 (2.6)	1.7 (2.0)	0	0
137	*Prevotella*	4.5 (3.2)	0.3 (0.6)	0.5 (1.8)	0.01 (0.03)	0.003 (0.01)
4	*Veillonella*	0.2 (0.2)	0.9 (0.5)	1.1 (0.3)	1.6 (0.6)	0.8 (0.4)
89	*Megasphaera*	0.6 (0.6)	2.6 (4.2)	0.6 (1.4)	0.8 (1.3)	0.06 (0.2)
30	*Veillonella*	0.1 (0.1)	1.7 (1.0)	1.7 (0.8)	0.6 (0.4)	0.2 (0.2)
36	Firmicutes	0.3 (0.3)	0.1 (0.2)	0.1 (0.2)	1.8 (0.7)	1.1 (0.5)
555	*Rothia*	3.5 (2.1)	0.001 (0.01)	0.001 (0.01)	0.001 (0.003)	0
201	*Prevotella*	2.5 (1.6)	0.1 (0.2)	0.01 (0.02)	0.01 (0.03)	0.002 (0.01)
22	*Veillonella*	0	0.002 (0.01)	0.4 (0.3)	0.001 (0.005)	2.0 (0.6)
16	*Pasteurellaceae*	0.6 (0.6)	0.9 (0.9)	0.6 (0.5)	0	0
1146	*Gemella*	2.0 (2.7)	0.04 (0.06)	0.03 (0.06)	0	0
194	*Solobacterium*	0.2 (0.1)	0.7 (0.8)	0.8 (0.8)	0.2 (0.3)	0.1 (0.2)
186	Firmicutes	0	0.003 (0.01)	0.2 (0.2)	0.001 (0.01)	1.7 (0.4)
74	*Veillonella*	0.1 (0.1)	0.5 (0.4)	0.4 (0.3)	0.4 (0.3)	0.2 (0.3)
44	*Veillonella*	0.1 (0.1)	0.3 (0.2)	0.3 (0.2)	0.4 (0.2)	0.4 (0.2)
477	*Granulicatella*	0.6 (0.3)	0.4 (0.3)	0.4 (0.3)	0.1 (0.1)	0.01 (0.03)
269	*Lactobacillus*	0	0	0.1 (0.1)	0.003 (0.01)	1.3 (0.7)
2	*Veillonella*	0.1 (0.2)	0.3 (0.1)	0.3 (0.1)	0.4 (0.1)	0.4 (0.2)

Reads from the microcosm samples (Figure 1) were dominated by a single phylum – Firmicutes, contributing 76 – 96% of reads per sample (average 88.7%, SD 5.4) if grown in the plain medium (Suc-). Sucrose supplementation to the growth medium (Suc+) increased the proportion of this phylum (93-100% reads/sample; average 98%, SD 1.5) significantly ($p<0.05$). Irrespective of the presence of sucrose, reads belonging to Actinobacteria, Bacteroidetes, Proteobacteria and candidate division TM7 decreased significantly ($p<0.05$) in the microcosms compared to the inocula. Fusobacteria remained around 2% of the reads/sample in microcosms grown in the plain medium. Growth in the presence of sucrose nearly eliminated representatives of Bacteroidetes and entirely excluded Fusobacteria and Proteobacteria from the microcosms. Reads classified as representatives of the candidate division TM7 (unculturable and unidentified bacteria) were found in 41% of the microcosms, with an average of 0.05% (range 0 – 0.3%; SD 0.08) of reads per sample. Higher prevalence of this bacterium was found in the microcosms grown in the plain medium (in 77% and 50% of microcosms, Suc-LGG- and Suc-LGG+, respectively), than in the microcosms grown in sucrose-supplemented medium (in 18% and 13% of the microcosms, Suc+LGG- and Suc+LGG+, respectively). Reads that could not be classified to any of the known phyla (Unclassified Bacteria, Figure 1) increased from 1.4% reads (SD 0.4) per sample in the inocula to 4% of reads (SD 1.3) per Suc- microcosm or decreased to 0.6% reads (SD 0.4) per Suc+ sample.

Firmicutes dominated all microcosm samples (Figure 1). The most abundant group (38 – 65% of all Firmicutes) was classified into the *Veillonellaceae* family (Figure 4). Reads from this family were clustered into 115 OTUs, 63 of which were classified as genus *Veillonella* (found in all groups of samples), 12 – as genus *Megasphaera* (in all groups of samples), two – as genus *Centipeda* (in inoculum and Suc- samples), one – genus *Selenomonas* (inoculum only), one – genus *Dialister* (in Suc- samples) with remaining 36 OTUs as unclassified *Veillonellaceae* (in all groups). Among the genus *Veillonella* OTUs, four OTUs were only found in the inocula, 10 OTUs – only in the Suc- microcosms, and two OTUs – only in the Suc+ samples. Two OTUs (#784 and #792) were highly prevalent (present in >80% of samples) in Suc- microcosms, but were absent from the Suc+ samples. However, none of the predominant OTUs found in the Suc+ groups were absent in the Suc- samples. Three OTUs (#22, #1636, #1841)

were more prevalent in the microcosms grown in the presence of probiotics (LGG+), irrespective of the presence of sucrose.

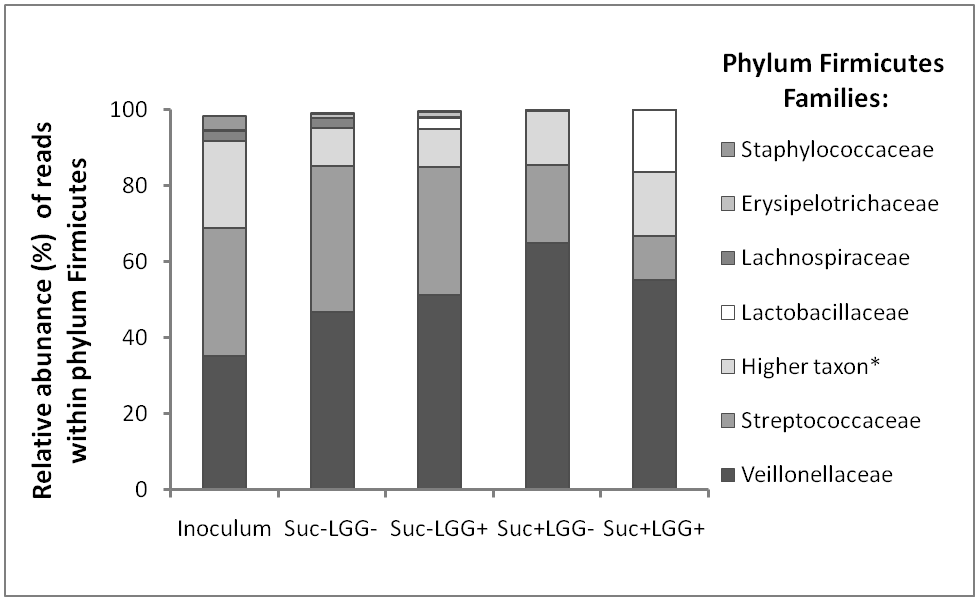

Figure 4. Distribution of reads (%) within phylum Firmicutes, classified at the Family level by sample types – inoculum and microcosms obtained from four different growth conditions: plain medium (Suc-LGG-), plain medium with LGG (Suc-LGG+), sucrose-supplemented medium (Suc+LGG-) and sucrose-supplemented medium with LGG (Suc+LGG+). * - reads that could not be classified at the family level but at higher levels are presented as one taxonomic group (Higher taxon).

Clustering of the microbiome profiles by principal component analysis (PCA)

The dataset with the 923 OTUs was log2 transformed and exposed to data reduction by PCA. First, the PCA was applied to the full dataset including the microcosms and inocula. The two major principal components together explained 50% of the total variance and resulted in clear clustering of microcosms by the growth condition (with or without sucrose and with or without LGG) and separated the inocula from the microcosms (Figure 5).

PCA analysis showed that the principal component 1 (PC1) discriminated between the inocula of the low MS group and the high MS group (Figure 6A) and explained 65% of the total variance. The five main loadings of this component belonged to the OTUs classified as *Porphyromonas*, *Neisseria*, Lactobacillales, *Prevotella* and *Gemella* (Figure 6B). Of these, low MS-inocula contained significantly higher proportion of *Porphyromonas* (OTU #336) and *Neisseria* (OTU #662), and significantly lower proportion of *Prevotella* (OTU #137, #201) than the high MS-inocula ($p<0.05$).

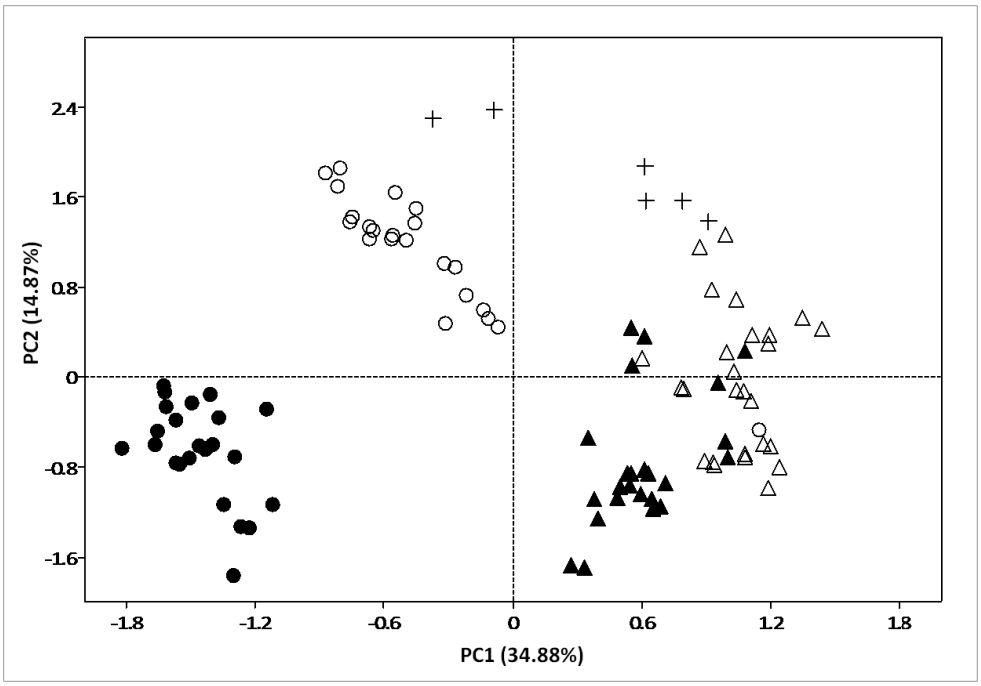

Figure 5. Principal Component Analysis (PCA) plot of the principal component 1 (PC1) and the principal component 2 (PC2) on the complete dataset (inocula and all microcosms) of the normalized and log2 transformed OTU data. Crosses indicate saliva samples used as inocula; open triangles – microcosm samples grown in the plain medium (Suc-LGG-); filled triangles – plain medium with LGG (Suc-LGG+); open circles – sucrose-supplemented medium (Suc+LGG-); filled circles – sucrose-supplemented medium with LGG (Suc+LGG+). PC1 explained 34.9%, PC2 – 15% of the total variance.

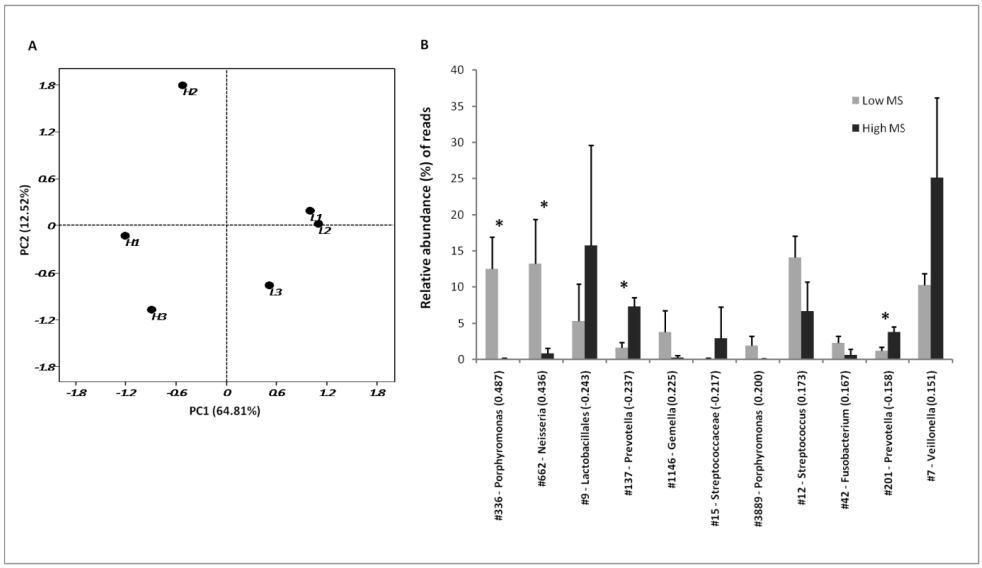

Figure 6. PCA on the inocula: (**A**) PCA plot of the first two components (PC1 and PC2) of the PCA (H1, H2, H3 - individual saliva with high mutans streptococci (MS) counts, L1, L2, L3 – saliva with low MS counts). PC1 (65% of variance) discriminated between the low MS and high MS salivas. (**B**) Relative abundance of those OTUs which contributed most to the PC1 (values in the brackets are the PC1 loadings of the respective OTUs). * - statistically significant difference between low and high MS samples ($p<0.05$; Independent samples T-test).

Next, we applied PCA to assess the effects of the microcosm source (high or low MS-inoculum) on the microcosms. In the plain medium, microcosms that originated from the low MS-inoculum were clearly separated from the high MS-microcosms by the PC1, explaining 38% of the total variance (Figure 7A). High MS Suc- microcosms harbored significantly higher proportion of the OTUs classified as *Megasphaera* (OTU #89), *Solobacterium* (OTU #194), *Oribacterium* (OTU #178) and *Prevotella* (OTU #72) than the low MS Suc- microcosms, while low MS Suc- microcosms had higher proportion of *Veillonella* (OTU #7) and *Streptococcus* (OTU #46) (Figure 7B). Among the microcosms grown in the sucrose-supplemented medium, PC1 separated the high and the low MS-microcosms and explained 42% of the variance (Figure 8A). High MS Suc+ microcosms harbored significantly higher proportion of Lactobacillales (OTU #9), *Megasphaera* (the same OTU as in Suc- microcosms) and

Actinomyces (OTU #70), while unclassified Firmicutes (OTU #18) were significantly more abundant in low MS Suc+ microcosms than in the high MS samples (Figure 8B).

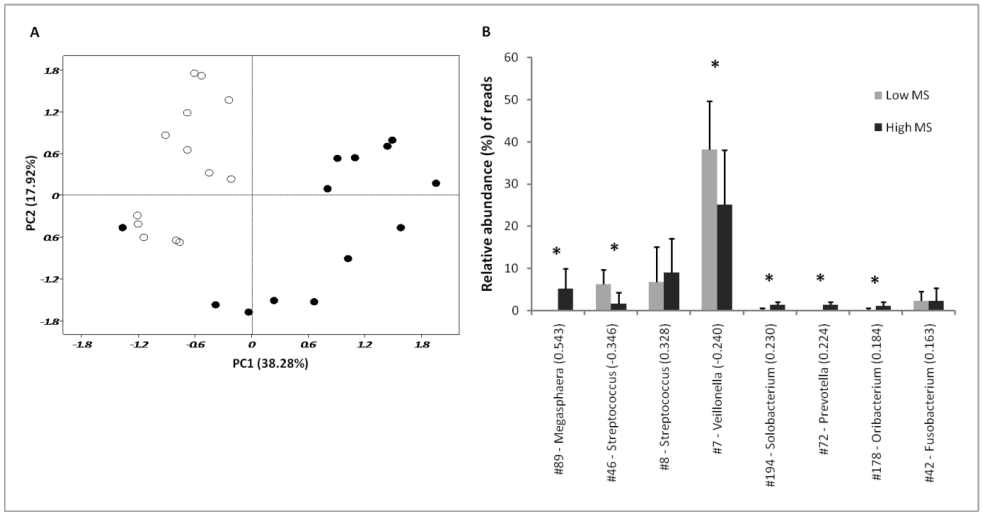

Figure 7. Effect of the type of the inoculum on the microcosm samples grown in the plain medium (Suc-LGG-): (**A**) the PCA plot with the first main components of the PCA. PC1 discriminated between samples derived from the low MS-inocula (open circles) and the high MS-inocula (filled circles). (**B**) Relative abundance of those OTUs which contributed most to the PC1 (values in the brackets are the PC1 loadings of the respective OTUs). * - statistically significant difference between low and high MS samples ($p<0.05$; Independent samples T-test).

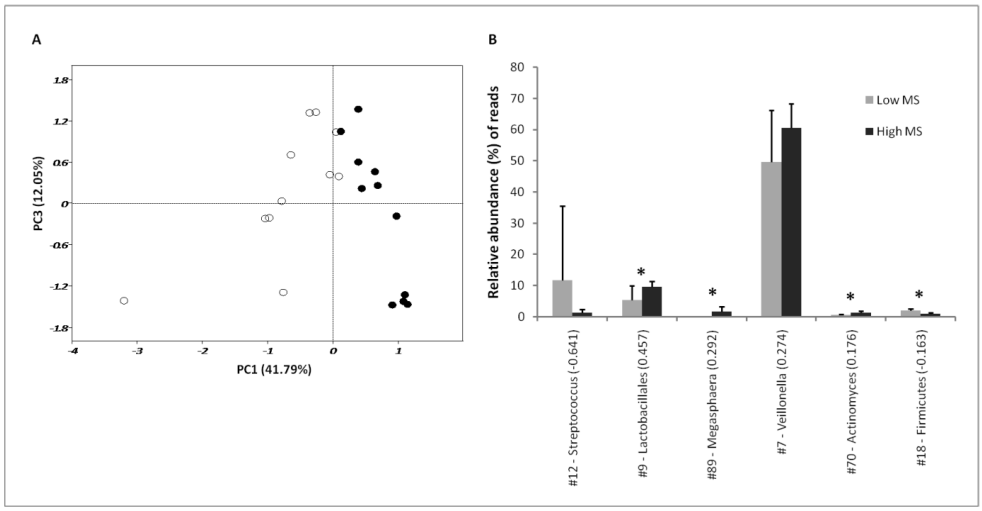

Figure 8. Effect of the type of the inoculum on the microcosm samples grown in the sucrose-supplemented medium (Suc+LGG-): (**A**) the PCA plot with the first main components of the PCA. PC1 discriminated between samples derived from the low MS-inocula (open circles) and high MS-inocula (filled circles). (**B**) Relative abundance of those OTUs which contributed most to the PC1 (values in the brackets are the PC1 loadings of the respective OTUs). * - statistically significant difference between low and high MS samples ($p<0.05$; Independent samples T-test).

Next, we assessed the effects of the sucrose supplementation in the growth medium on the microbiome profiles of the microcosms (Figure 9). In both, the low and the high MS-inoculum derived microcosms, the first principal component (PC1) discriminated the plain medium grown samples from the sucrose-supplemented samples and explained about 50% of the total variance (Figure 9A, B). Unclassified Lactobacillales (OTU #9), unclassified Firmicutes (OTU #36) and *Veillonella* (OTU #95) increased significantly with the sucrose supplementation; while *Streptococcus* (OTU #12) and *Fusobacterium* (OTU #42) were more abundant in the plain-medium (Suc-) microcosms irrespective of the type of the inoculum (Figure 9C, D). Only in the low MS inoculum group, *Streptococcaceae* (OTU #15) increased and *Streptococcus* (OTU #46) decreased significantly in Suc+ compared to Suc- samples (Figure 9C). Specific to the high MS group was the increase of *Veillonella* (OTU #7) and decrease of *Megasphaera* (OTU #89), *Prevotella* (OTU #72) and *Oribacterium* (OTU #178) in Suc+ samples compared to Suc- group (Figure 9D).

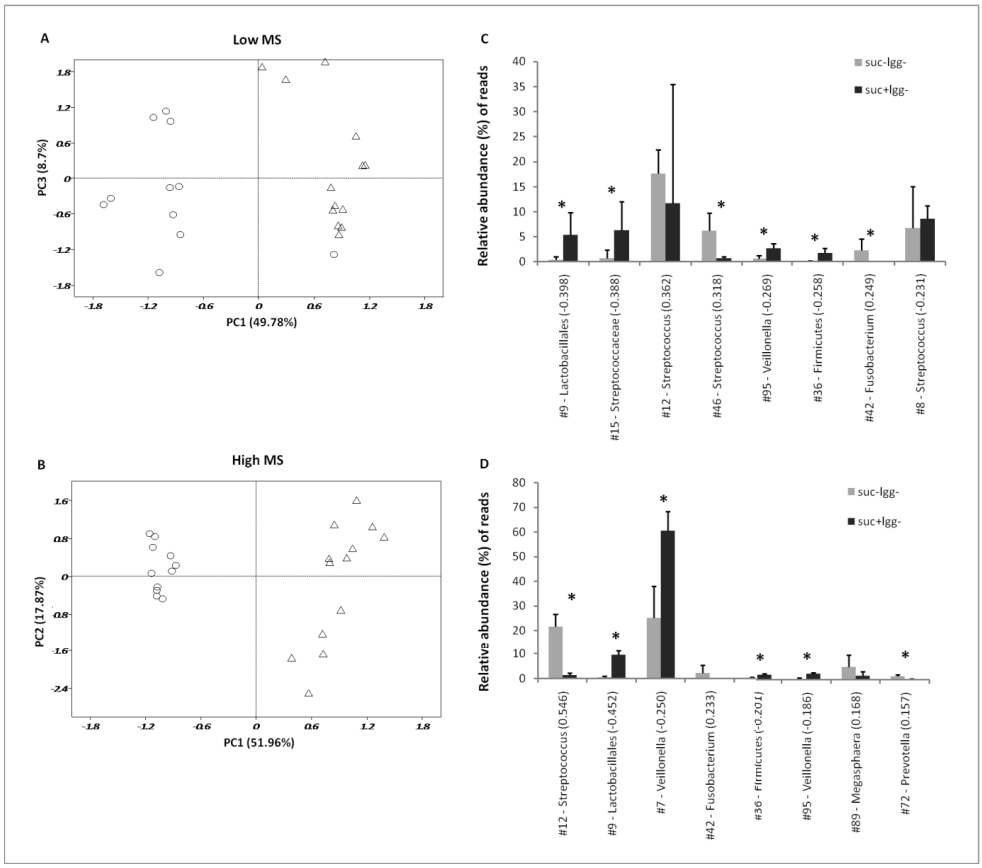

Figure 9. The effects of the sucrose-supplement on microcosms per type of the inoculum (low or high MS in saliva): (**A**, **B**) PCA plots with the first two components of the PCA. PC1 discriminated between samples grown in the plain medium (Suc-LGG-) marked as triangles and samples grown in sucrose-supplemented medium (Suc+LGG-) marked as circles. (**C**, **D**) Relative abundance of those OTUs which contributed most to the PC1 (values in the brackets are the PC1 loadings of the respective OTUs) in samples inoculated with low MS-saliva (**C**) and high MS-saliva (**D**). * - statistically significant difference between low and high MS samples ($p<0.05$; Independent samples T-test).

Finally, we addressed the effects of the probiotic strain *Lactobacillus rhamnosus* GG (LGG) on the microbiome profiles. To avoid dichotomization of the samples in LGG- and LGG+ groups entirely due to the high abundance of the probiotic LGG in the sample (26% (SD 14) and 57% (SD 15) of *L. rhamnosus* 16S rDNA proportion against total 16S rDNA, in Suc- and Suc+ microcosms, respectively, as reported in Pham *et al*.

(PHAM *et al.* 2011), we excluded single predominant OTU that was identified as LGG (OTU #94: 2% of reads in Suc-LGG+ microcosms, 15% of reads in Suc+LGG+ microcosms) from the raw dataset, normalized the data without this OTU, log2 transformed the data and performed the PCA (Figure 10). The effects of the LGG on the plain medium (Suc-) microcosms were minimal (data shown in supplementary file 3) and none of the PCA components discriminated the effect of LGG. In sucrose-supplemented microcosms, however, PC1 of the PCA discriminated clearly between LGG- and LGG+ samples, both in low (37% of variance) and in high (45% of variance) MS-inocula derived groups of samples (Figure 10A, B). Irrespective of the inoculum type, microcosms with LGG supplementation showed significant increase in unclassified Firmicutes (OTUs #186; #50), *Lactobacillus* (OTU #269), *Veillonella* (OTUs #22; #124), Lactobacillales (OTUs #255; #52), Bacilli (OTU #197) and decrease in *Streptococcus* (OTU #12) (Figure 10C, D). Specific to the high MS-group of samples was the decrease of unclassified *Streptococcaceae* (OTU #15) and genus *Megasphaera* (OTU #89) with LGG (Figure 10D).

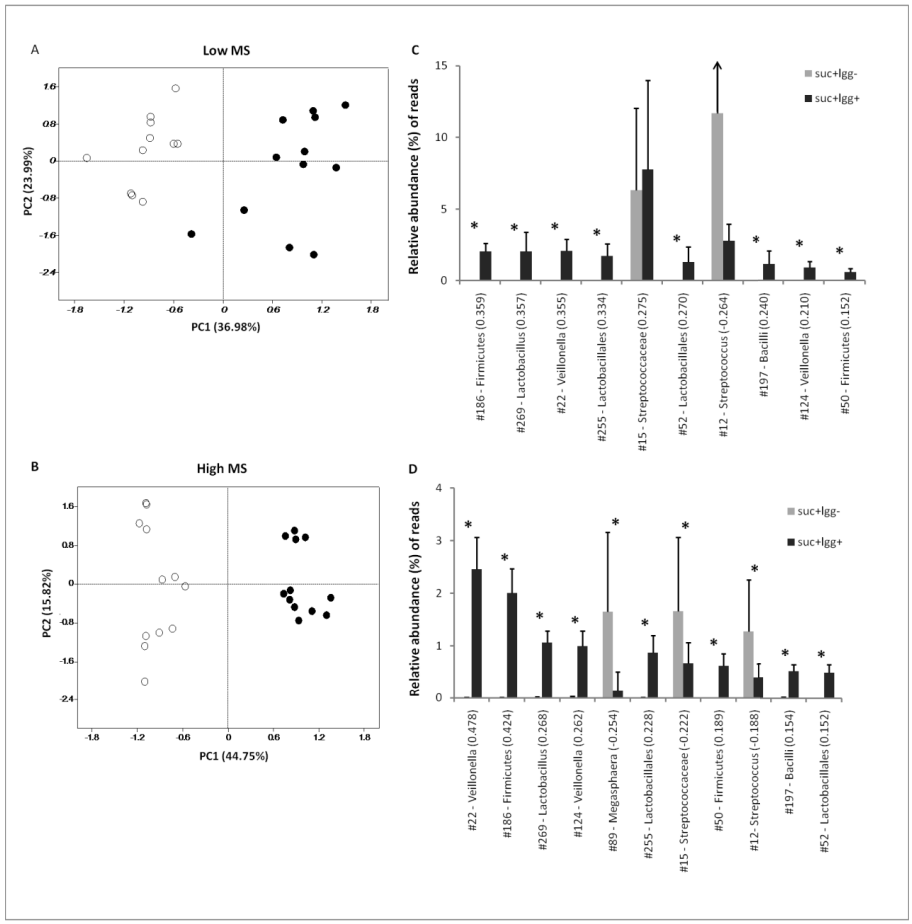

Figure 10. Effects of the probiotic strain *Lactobacillus rhamnosus* GG (LGG) on the microcosms** grown in sucrose-supplemented medium per type of the inoculum (low or high MS in saliva): (**A**, **B**) PCA plots with the first two components of the PCA. PC1 discriminated between samples grown without LGG (Suc+LGG-) marked as open circles and samples grown with LGG (Suc+LGG+) marked as filled circles. (**C**, **D**) Relative abundance of those OTUs which contributed most to the PC1 (values in the brackets are the PC1 loadings of the respective OTUs) in samples inoculated with low MS-saliva (**C**) and high MS-saliva (**D**). * - statistically significant difference between low and high MS samples ($p<0.05$; Independent samples T-test). ** - Before this PCA analysis, OTU #94 (BLAST hit as *L. rhamnosus* and highly abundant in the samples with LGG supplementation, while absent in LGG- group) was removed from this dataset to prevent that the presence of this OTU splits the microbial profiles LGG-/LGG+ groups. For improved visualization of the graph 10C, the high standard deviation (SD 37) of average relative abundance of the OTU #12 is not shown and is indicated by an arrow.

Comparison between MLPA and pyrosequencing outcome

Of the microcosm samples that were analyzed in the current study with 454 pyrosequencing, 92 samples have also been assessed with multiplex ligation-dependent probe amplification (MLPA) technique (PHAM *et al.* 2011). For the MLPA, we used a probe panel consisting of 20 probes, of which 18 were species-specific, one – genus specific probe targeting genus *Prevotella*, and one probe was specific for yeast species *Candida albicans*. To compare the microbial profiles of the two methods, we used the higher taxon data (genus or more inclusive taxon) that included 64 taxa (supplementary file 1) from the pyrosequencing output. Two main components of the PCA explained about 60% of the total variance in both datasets (Figure 11A, B). The PCA of the pyrosequencing data separated all six inocula of the microcosms from the microcosm groups and clustered the plain-medium grown microcosms (triangles in Figure 11A) from the microcosms grown with sucrose supplement (circles in Figure 11A), while the PCA on the MLPA data showed stronger discrimination between the LGG-containing groups (filled symbols in Figure 11B) and the microcosms without LGG (open symbols) than the sequencing data.

Relative abundance of genus *Prevotella*-specific probe in the MLPA dataset correlated significantly (Spearman's rho 0.836; $p<0.001$) with the proportion of reads classified as *Prevotella* in the pyrosequencing dataset (Figure 12). The probability of the pyrosequencing method to detect *Prevotella* in the sample (sensitivity of the method) was 0.72, while the specificity (probability that the sample does not contain any *Prevotella*) of the pyrosequencing was 0.86 against the MLPA (Table 2). All but two of the 14 false negatives of the pyrosequencing method were found at a low proportion (0.1 to 1%) in the MLPA samples. The remaining two samples with no reads classified as *Prevotella* contained 4.8 and 8.3% of *Prevotella* according to the MLPA. False positive samples (N=6) of the pyrosequencing all were found at a very low proportion (0.003 – 0.025% of the reads).

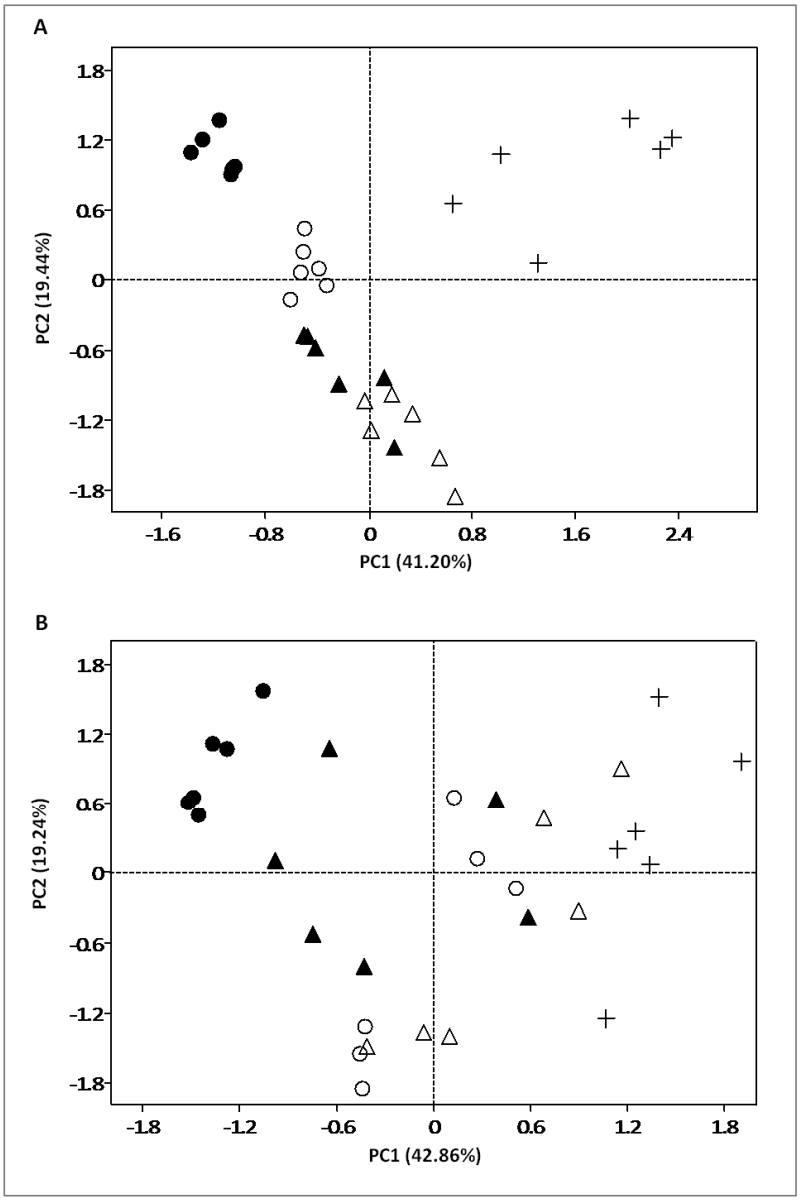

Figure 11. PCA plots of the first two principal components (PC1 and PC2) of (**A**) the final taxon data (64 taxa at genus or higher level) obtained by pyrosequencing and (**B**) by MLPA using 20 MLPA probe-set (PHAM *et al.* 2011). Symbols in the plots: cross – inoculum, open triangle – Suc-LGG-; filled triangle – Suc-LGG+; open circle – Suc+LGG-, filled circle – Suc+LGG+.

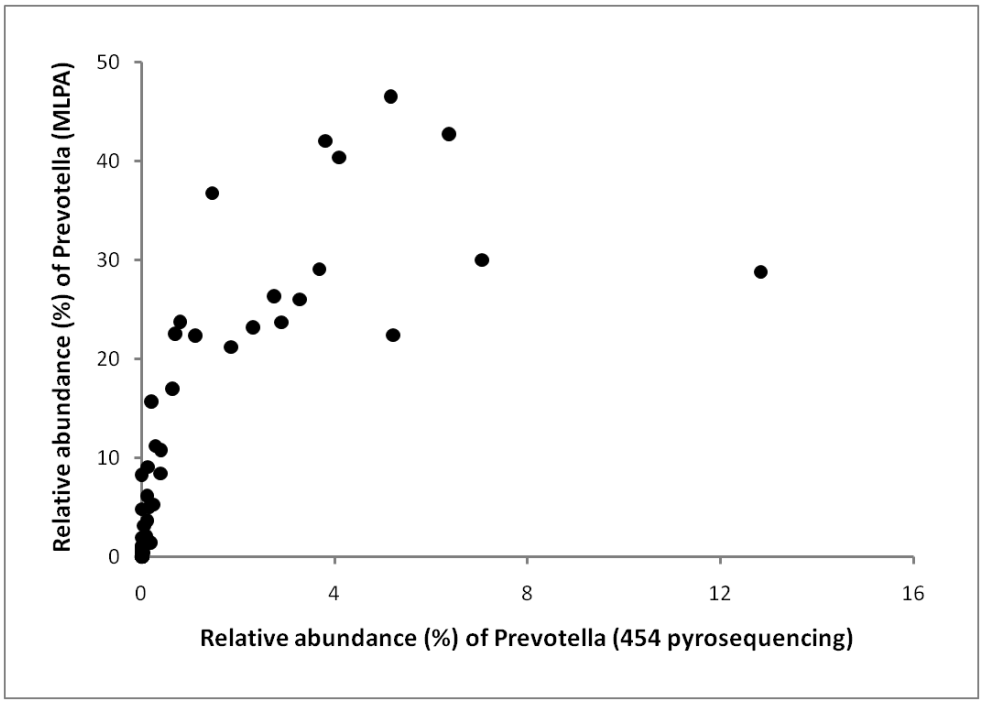

Figure 12. The relative abundance of genus *Prevotella* specific MLPA-probe by the relative abundance of genus *Prevotella* as a final taxon in the 454 pyrosequencing dataset per individual sample (N=92). Correlation was statistically significant (Spearman's rho 0.836; p<0.001).

Table 2. Detection of genus *Prevotella* in the microcosms (N=92) by MLPA and by pyrosequencing.

Prevalence of *Prevotella* in microcosms		Pyrosequencing	
		+	-
MLPA	+	36	14
	-	6	36

DISCUSSION

This is, to our knowledge, the first time that effects of inoculum and different growth conditions on saliva-derived microcosms have been assessed by the 454 pyrosequencing approach. This comprehensive parallel sequencing of short fragments of the 16S rDNA gene allowed a non-targeted, open-ended profiling of complex microbial communities of unknown composition.

Pre-screening of the saliva donors for culturable mutans streptococci (MS) allowed us to dichotomize the inocula into low and high MS group. The two groups showed marked differences already at the phylum level. OTU-profile analyses by the principal components confirmed the differences between the two types of inocula. The major differences were in the relative abundance of reads classified as *Neisseria*, *Porphyromonas* (all significantly more abundant in the low MS-inocula) versus *Prevotella*, which was more abundant in the high MS-inocula. Surprisingly, none of the significantly different OTUs could be classified as *Streptococcus mutans* while both culturing on a selective plate and performing a specific qPCR probe counts showed significantly higher mutans streptococci abundance in the high MS group (PHAM *et al.* 2011). It could be due to a relatively low proportion of this organism in saliva in combination with a high sensitivity of targeted methods, or due to the incomplete reference sequences in publicly available 16S rDNA databases.

The microbial profiles of the saliva used as inocula were typical for the salivary microbiome when compared to the salivary profiles obtained in our previous studies using pyrosequencing (KEIJSER *et al.* 2008; ZAURA *et al.* 2009) both by the predominant taxa and by the relative abundance of these taxa. Microcosms, however, differed significantly from the inocula, with representatives of the phylum Firmicutes dominating in all samples. This finding was not surprising because of the selectivity of the growth media employed to simulate saliva (TIAN *et al.* 2010). Tian and colleagues (TIAN *et al.* 2010) performed elaborate series of experiments in search of the "perfect" medium to sustain the original oral communities *in vitro*. The aim of the experiments in Pham *et al* (PHAM *et al.* 2011), however, was not to sustain the original community in all its complexity but to address the effects of probiotic strain *Lactobacillus rhamnosus* GG (LGG) on the composition, hence cariogenicity, of the

microcosms. Therefore we used a relatively simple medium (McBain *et al.* 2005) which supports the growth of the phylum Firmicutes and thereby induces a severe cariogenic challenge mainly by sucrose- and lactate-metabolizing members of the *Streptococcaceae*, *Lactobacillaceae* and *Veillonellaceae* family of phylum Firmicutes.

Interestingly, the microcosms grown without sucrose supplementation showed a double number of taxa (OTUs) compared to the original inocula or to the microcosms grown with sucrose supplementation. In total, 272 OTUs, though at low abundance, were exclusively found in these plain medium microcosms. The artificial saliva we used must have supported the growth of diverse groups of salivary microorganisms that were below the detection limit in the inoculum, but could establish itself in the 72-h biofilms grown without the caries-inducing challenge. Another interesting finding was the ability of the representatives of candidate division TM7 to establish in a majority of the plain-medium microcosms and to a lesser degree, in the sucrose-supplemented microcosms. TM7 is a diverse bacterial division (equivalent to a phylum but so far uncharacterized), found in terrestrial, aquatic and clinical habitats, and is known only from environmental 16S rDNA sequence data without any cultivated members (Hugenholtz *et al.* 2001; Ouverney *et al.* 2003). It has been associated with mild periodontitis (Brinig *et al.* 2003) and with active inflammatory bowel disease (Kuehbacher *et al.* 2008).

Addition of sucrose to the growth medium introduced a severe cariogenic challenge: the pH of the spent medium decreased from pH 7 to pH 5, and the dentin substratum onto which the microcosms were grown showed substantial mineral loss (Pham *et al.* 2011) irrespective of the inoculum (high or low MS group) for the microcosms. Diversity of the cariogenic microcosms was significantly lower than the diversity of the plain-medium communities, supporting our previous findings (Pham *et al.* 2009) and clinical reports (Li *et al.* 2007; Li *et al.* 2005). The cariogenic communities of this study were dominated by *Veillonellaceae* family members.

S. mutans has been associated with dental caries (Loesche 1986; Loesche *et al.* 1975) and the counts of MS in saliva are used in caries risk assessments (Zhang *et al.* 2007). In Pham *et al.* (Pham *et al.* 2011) we hypothesized that in the absence of *S. mutans* other cariogenic microorganisms must have become abundant under these highly

cariogenic conditions. The comparison of the microcosms evolving from the low and the high MS inocula showed that these two groups formed two distinguishable microbial profiles. The major difference was in the increase of unclassified *Streptococcaceae* (OTU #15) and decrease of one representative of genus *Streptococcus* (OTU #46) in the low MS communities, and the decrease of *Megasphaera* (OTU #89), *Prevotella* (OTU #72) and *Oribacterium* (OTU #178) in the high MS microcosms in the presence of sucrose. Based on these differences, the OTU #15 may have been involved in cariogenic activity of the low MS microcosms grown with sucrose supplementation.

The OTUs that increased in proportion in both (low and high MS) microcosm types if exposed to sucrose, were unclassified Lactobacillales, unclassified Firmicutes and representatives of genus *Veillonella*. The presence of sucrose promoted the growth of *Veillonella* (OTU #7 in the high MS group and OTU #95 in the both MS groups). The OTU #7 was highly dominant in the low MS group samples grown without sucrose (on average, 40% of the reads) and it increased non-significantly in the presence of sucrose (about 50% of reads), while this particular OTU increased in abundance from 25% (plain medium) to 60% (sucrose-supplemented medium) of the reads in the high MS group. Veillonellae and streptococci are metabolically linked through streptococcal fermentation of sugars to lactic acid, which is a carbon source for the nonsaccharolytic veillonellae with acetate as the main metabolic endproduct (CHALMERS *et al.* 2008; DELWICHE *et al.* 1985). For this reason veillonellae are considered non-cariogenic microorganisms. However, both acetic and lactic acid and their buffers will induce demineralization of enamel and dentin. The rate of demineralization increases with decreasing pH. Considering buffering in the oral cavity, demineralization typically occurs at pH values corresponding to the pK values of the acids present. Acetic acid has a pK value of 4.5, and for lactic acid it is one pH unit lower. Dentin, as used in this study, has a higher solubility than enamel, and this tissue will start to dissolve already around pH=6 (HOPPENBROUWERS *et al.* 1987). This pH is reached quickly when acids are formed in the dental plaque biofilm. In addition, it has been shown that acetate, unlike lactate, penetrates and thereby disappears into porous dental tissue such as dentin (GEDDES *et al.* 1984). Since lactate breakdown into acetate would not be inhibited by a build-up of acetate,

veillonellae species would benefit from this process and thus would have an ecological advantage. Recent clinical studies have demonstrated the association of *Veillonella* species with severe early childhood caries (BECKER *et al.* 2002; KANASI *et al.* 2010; MARCHANT *et al.* 2001), deep dentinal lesions (LIMA *et al.* 2011), as well as with early and established cavities in children (AAS *et al.* 2008; LING *et al.* 2010). Kanasi *et al.* (KANASI *et al.* 2010) explained the association of *Veillonella* with severe dental decay by a lactate-rich environment of the carious dental tissue, and proposed for veillonellae a key role in supporting the biofilm of carious infection.

To assess the effects of probiotic strain *Lactobacillus rhamnosus* GG (LGG) on the microcosms, we removed a single predominant OTU (OTU #94) that we identified as *L. rhamnosus* prior to principal component analysis (PCA). The PCA showed that addition of the LGG to the inoculum affected the microcosms that were grown in the presence of sucrose, but not in the plain medium. A potential explanation for this difference might be in the low relative abundance of LGG that was found in the plain-medium microcosms compared to the sucrose-supplemented group (PHAM *et al.* 2011). Although LGG does not ferment sucrose, it preferred growth at pH 5 compared to pH 7. In the presence of LGG, several other Firmicutes (*Veillonella*, *Lactobacillus* and unclassified members of Lactobacillales, Bacilli and Firmicutes) increased or decreased (*Streptococcus*, *Streptococcaceae*, *Megasphaaera*) in their relative abundance. The taxonomic resolution of the current pyrosequencing reads (on average 370 nt, spanning over the hypervariable region V5-V7 of 16S rDNA) and the limitations of publicly available 16S rDNA reference databases (*e.g.*, over-representation of cultured and isolated microorganisms; erroneous sequences due to lack of continuous and updated curation) did not allow us to identify the involved OTUs. One of the OTUs (#15, unclassified *Streptococcaceae*) that reduced in abundance in high MS microcosms supplemented with sucrose and LGG, was significantly more abundant in the high MS inocula, and increased in abundance in low MS-inocula derived microcosms if grown with sucrose supplementation. We could not confirm that this OTU is *Streptococcus mutans* (BLAST search resulted in alignment at 96% identity with *Streptococcus mutans* UA159 sequence), but could not exclude this probability either. To clarify this we may need to sequence numerous

clinical isolates of *S. mutans* and to develop a custom reference database of 16S rDNA sequences of this clinically relevant microorganism.

In our previous study we applied the multiplex ligation-dependent probe amplification (MLPA) technique to profile the same set of experimental and salivary samples with 20 specific probes (PHAM *et al.* 2011). The comparison of both techniques indicates that targeting only limited number of microorganisms does not cluster the experimental groups as clearly as the open-ended approach by pyrosequencing. Nevertheless, we could confirm the effects of the individual inocula (high and low MS saliva), plain and sucrose-supplemented medium and the addition of LGG on microcosms using either of the methods. The presence of the genus *Prevotella*-specific probe in the MLPA probe panel allowed us to correlate the relative proportion of this probe in the samples with the proportion of the genus *Prevotella* taxon in the pyrosequencing dataset of the same samples. In 78% of the samples both methods agreed on the presence or absence of *Prevotella*. Sensitivity of the pyrosequencing method is highly dependent on the sequencing depth. In the current study individual samples gave between 1,277 and 8,261 reads (average 3,863). A single read classified as *Prevotella* would contribute to 0.01 - 0.08% (average 0.03%) of the reads of the individual sample. In the samples where *Prevotella* was prevalent by both methods, we found between 0.015% and 12% (average 1.9%) reads/sample classified as *Prevotella*. This clearly shows that by increasing of the number of reads per sample we are able to increase the sensitivity of the pyrosequencing method. In two samples, we found relatively high proportion (4.8% and 8.3%) of *Prevotella* by MLPA but did not find such presence by pyrosequencing. This could be explained by the methodology: MLPA probe of genus *Prevotella* was designed to target exclusively this genus (nt position 579 – 636 of *E. coli* 16S rDNA), while with pyrosequencing all microorganisms were targeted (nt position 785 – 1175 of *E. coli*). For pyrosequencing, the sequenced part of 16S rDNA covers three hypervariable regions (V5-V7) flanked by conserved sequences. In cases where taxonomical classification could not be assigned to the genus level with at least 80% probability, the sequences were assigned to a higher taxon (family, order, class or phylum). This may have resulted in the observed differences at the genus level.

The use of the open-ended microbial profiling approach, 454 pyrosequencing, allowed us to disclose the differences between natural microbial communities (individual salivas) and their *in vitro* evolved counterparts - microcosms. We observed clear differences in the microbial profiles between the two types of inocula (low and high mutans streptococci counts in saliva), supporting the ecological plaque hypothesis. We showed that microcosms derived from these two types of predefined inocula remained distinct at different growth conditions. We conclude that selection of the inoculum for microcosm experiments should be based on defined criteria, which would depend on the aim of the study. The low taxonomic resolution of the pyrosequencing sequences limited the characterization of the microcosms to the OTU level or to higher taxa with maximum resolution at the genus level. This limitation, however, could be improved by the use of a custom-made and curated reference sequence database. Comparison of targeted profiling by MLPA with high throughput sequencing by 454 pyrosequencing showed that the MLPA is a valid method in microcosm profiling. The sequences generated by the open-ended approach will allow relevant probe selection for less costly, targeted community profiling methods.

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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

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.

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SAMENVATTING

Dit proefschrift is een onderdeel van het onderzoeksproject "Probing Dynamic Microbial Community Interactions" binnen de Nederlandse Technologiestichting STW. Het uiteindelijke doel van dit onderzoek was het verwerven van een algemeen inzicht in het dynamische samenspel tussen omgevings- en gastheerfactoren en microbiële ecosystemen, en om te begrijpen hoe dit kan leiden tot een gezonde mond, of juist het ontstaan van ziekte.

Voor onderzoek naar microbiële ecosystemen is het van cruciaal belang om te beschikken over een '*high throughput*' methode om micro-organismen te identificeren en kwantificeren. Bij het gebruik van enkel fenotypische testen worden isolatie en identificatie van micro-organismen gelimiteerd tot slechts een aantal species, waaronder de meest bekende pathogenen.

Het doel van het project in dit proefschrift was daarom om een makkelijke, kostenefficiënte en nauwkeurige techniek te ontwikkelen om *high throughput* screening van de samenstelling en dynamiek van het microbiële ecosysteem van de mond mogelijk te maken.

De focus lag daarbij met name op de gevolgen van een verstoring door twee specifieke stammen van *Lactobacillus,* namelijk *L. salivarius* W24 (hoofdstuk II) en *L. rhamnosus* GG (hoofdstuk IV en V), voor het orale microbiële ecosysteem, met betrekking tot de samenstelling, de productie van zuren, en het cariogene potentieel.

Als model voor een complex oraal microbieel ecosysteem (hoofdstuk II, III, en IV) werden '*microcosms*' uit speeksel gebruikt. Voor de *high throughput* screening van de samenstelling van deze complexe orale biofilms is een gerichte methode ontwikkeld: multiplex-dependent ligation probe amplification (MLPA). In de hoofdstukken III, IV en V worden de toepasbaarheid en toetsing van deze methode voor karakterisering van deze *microcosms* behandeld.

In **hoofdstuk II** is met behulp van de DGGE (denaturing gradient gel electrophoresis) fingerprinting-methode onderzocht hoe een *microcosm* uit speeksel kon worden verstoord door de probiotische darmbacterie *L. salivarius* W24. Dit

probioticum wordt aan sommige commerciële producten toegevoegd om de gezondheid van het maag- en darmstelsel te verbeteren. Het bleek dat *L. salivarius* W24 zelf geen biofilms kon vormen, en ook niet kon integreren in reeds bestaande biofilms. Wel kon W24 zich vestigen in een *microcosm* uit speeksel wanneer W24 en het speeksel tegelijk werden geïnoculeerd. Na deze integratie in het microbiële ecosysteem, kon *L. salivarius* W24 de ecologie van complexe biofilms die groeiden in de aanwezigheid van sucrose beïnvloeden: de pH werd verlaagd, en de microbiële diversiteit was verminderd. Deze resultaten suggereren dat dit specifieke probioticum het cariogene potentieel van het orale microbiële ecosysteem kan verhogen wanneer het zich zou kunnen vestigen in de mond. Verder is met deze studie ook aangetoond dat een relatief eenvoudige test, waarbij een probioticum en een complexe orale biofilm met elkaar in contact komen, gebruikt kan worden om te onderzoeken of een probioticum eventueel schadelijke effecten kan hebben op het microbiële ecosysteem van de mond.

In **hoofdstuk III** is de ontwikkeling en toepasbaarheid beschreven van de MLPA techniek voor de relatieve kwantificering van bacteriële species in complexe biofilms. MLPA is een nieuwe methode om verschillende genetische afwijkingen te detecteren, ontwikkeld in Amsterdam door wetenschappers van MRC-Holland in samenwerking met de Vrije Universiteit.

Om een aantal representatieve en bekende orale micro-organismen te identificeren op zowel geslacht en species niveau werd een set van specifieke MLPA-probes ontworpen. Met behulp van deze probes konden de verwachte verschillen in samenstelling van het orale microbiële ecosysteem zichtbaar worden gemaakt. Na het ontwerpen van de benodigde specifieke probes, was MLPA een snelle, eenvoudige, voordelige, en zeer gevoelige methode waarbij onderscheid kon worden gemaakt tussen sequenties die in slechts één nucleotide verschillen.

In **hoofdstuk IV** is de interactie tussen de probiotische darmbacterie *Lactobacillus rhamnosus* GG (LGG) en de cariogene mondbacterie *Streptococcus mutans* bestudeerd in een biofilm bestaande uit deze twee species. Ook is onderzocht of LGG invloed

had op het cariogene potentieel en de microbiële samenstelling van *microcosms* uit speeksel.

LGG had een remmend effect op de groei van streptokokken, zowel in een biofilm van twee species, als in *microcosm*s. LGG had echter geen significant effect op het cariogene potentieel van de *microcosms*. Hieruit kunnen wij concluderen dat LGG een niet-cariogeen probioticum is.

Verder werd aangetoond dat de MLPA methode geschikt is voor het aantonen van veranderingen in de samenstelling van *microcosms*, die zijn veroorzaakt door verschillende groeimedia, door verschillen tussen individuele speekselmonsters, en door verstoringen met het probioticum LGG.

In **hoofdstuk V** zijn de resultaten van de MLPA methode vergeleken met de uitkomsten van de *high throughput* 454 pyrosequencing methode, toegepast op dezelfde monsters. De resultaten van MLPA en 454 pyrosequencing waren vergelijkbaar wat betreft het onderscheiden van de effecten van inoculum en kweekcondities op de samenstelling van een microbieel ecosysteem. Dit bevestigt dat MLPA een valide methode is om complexe biofilms te karakteriseren. Bovendien kunnen met behulp van de resultaten van de 454 pyrosequencing methode een aantal relevante probes worden geselecteerd voor verdere analyses.

Al tientallen jaren ligt binnen onderzoek naar infectieziektes en ziektes van de mond de focus voornamelijk op slechts een enkel pathogeen. Recente ontwikkelingen suggereren echter sterk dat de micro-organismen in tandplaque als een gemeenschap functioneren, en niet als afzonderlijke individuen.

Methoden om microbiële ecosystemen te karakteriseren, zoals de MLPA methode met specifieke probes die werd ontwikkeld gedurende dit project, en de pyrosequencing methode die werd gebruikt ter validatie van MLPA, zullen daarom onmisbaar worden voor onderzoek naar de gezondheid van de mond. Dergelijke tools om microbiële ecosystemen te karakteriseren kunnen op vele manieren worden toegepast. Zo kunnen ze worden gebruikt om de effecten van behandelingen op de microbiële samenstelling te volgen, de aaneenschakeling van microbiota gedurende

de ontwikkeling van cariës en parodontitis te bestuderen, of het risico op mondziekten te beoordelen.

In de nabije toekomst zal niet meer alleen de aanwezigheid van micro-organismen, maar ook hun activiteit worden onderzocht, bijvoorbeeld door gebruik te maken van probes gebaseerd op mRNA sequenties, of door sequencing van het complete RNA van een microbioom of zelfs een volledig metagenoom. Tegelijk met deze nieuwe ontwikkelingen zullen ook nieuwe uitdagingen ontstaan op het gebied van gegevensverwerking en de interpretatie van de resultaten. Maar, uiteindelijk zullen dergelijke ontwikkelingen de kloof dichten tussen de nu beschikbare kennis en ons uiteindelijke doel: inzicht verkrijgen in dynamische interacties binnen microbiële ecosystemen, inzichten die de basis zullen vormen voor een fundamenteel begrip, en aanpak, van de problemen die complexe microbiële ecosystemen kunnen veroorzaken.

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CURRICULUM VITAE

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