Microbial detection strategies in supragingival plaque samples and possible dietary influences

Master's thesis

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Abstract

The human body hosts a large variety of microorganisms in high numbers. Understanding the characteristics of these microorganisms and their interactions with the human body is the topic of the Human Microbiome Project.

Following the same goals, this study was conducted to understand the interactions between microorganisms in the human oral cavity and inflammatory processes related to rheumatism. Previous findings suggest a correlation between periodontitis and the occurrence or severity of rheumatoid arthritis.

In order to investigate this hypothesis, a suitable detection method for several periodontal pathogens needed to be defined. The microorganisms in question were: *Aggregatibacter actinomycetemcomitans*, *Campylobacter rectus*, *Eikenella corrodens*, *Porphyromonas gingivalis*, *Prevotella denticola*, *Prevotella nigrescens*, *Streptococcus mutans*, *Tannerella forsythia* and *Treponema denticola*.

Supragingival plaque samples were analysed by real-time PCR and the obtained results cross-checked by agarose gel electrophoresis. Except for *C. rectus* and *P. nigrescens*, all microorganisms were detected with two different primer systems.

In almost all cases, the results obtained by real-time PCR were confirmed by the agarose gel electrophoresis. The method is to be modified though, in case quantitative results are desired. For the detection of the microbiome present in the human oral cavity, it is advised to use a method that allows high throughput at low costs.

Zusammenfassung

Der menschliche Körper beherbergt eine hohe Vielfalt an Mikroorganismen in großer Anzahl. Das „Human Microbiome Project“ hat sich die Untersuchung dieser Mikroorganismen und ihrer Interaktionen mit dem menschlichen Körper zum Ziel gesetzt.

Ganz in diesem Sinne wurde die folgende Untersuchung unternommen, um mögliche Zusammenhänge zwischen den Mikroorganismen in der menschlichen Mundhöhle und Entzündungsprozessen im Rahmen rheumatischer Erkrankungen festzustellen. Frühere Studien indizieren eine Korrelation zwischen Periodontitis und dem Auftreten bzw. dem Schweregrad von rheumatoider Arthritis.

Um dieser Hypothese auf den Grund zu gehen, sollte eine geeignete Untersuchungsmethode für verschiedene periodontalpathogene Keime gefunden werden. Die betreffenden Mikroorganismen waren folgende: *Aggregatibacter actinomycetemcomitans*, *Campylobacter rectus*, *Eikenella corrodens*, *Porphyromonas gingivalis*, *Prevotella denticola*, *Prevotella nigrescens*, *Streptococcus mutans*, *Tannerella forsythia* und *Treponema denticola*.

Supragingivale Plaque Proben wurden mittels real-time PCR untersucht und diese Ergebnisse jeweils anhand einer Agarose-Gelelektrophorese überprüft. Bis auf *C. rectus* und *P. nigrescens* wurden alle Mikroorganismen mit zwei unterschiedlichen Primer Systemen untersucht.

In fast allen Fällen wurden die Ergebnisse der real-time PCR durch die Gelelektrophorese bestätigt. Es ist jedoch eine Abwandlung der Methode erforderlich, falls quantitative Ergebnisse erwünscht wären. Um das Mikrobiom der menschlichen Mundhöhle zu erforschen sind Methoden mit hohem Durchsatz und niedrigen Kosten zu empfehlen.

Acknowledgement

“Was lange währt, wird endlich gut.” (Ovid)

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1 Introduction

1.1 Human microbiome project

The human body is the habitat for a large number of bacterial species. Microorganisms live on the surface and inside of our bodies. The "Human Microbiome Project" aims to study these microorganisms and their interactions. The main goal is to get an idea of the healthy human microbiome in five major areas and make these findings available to the scientific community. (Gevers, Knight et al. 2012)

The oral microbiome, or oral microbiota or oral microflora, are all microorganisms found in the human oral cavity and its contiguous extensions until the distal esophagus. (Dewhirst, Chen et al. 2010)

The samples are taken from one or more sites of different body regions: gastrointestinal tract, oral cavity, airways, skin and vagina. In the oral cavity for example, examined sites are the buccal mucosa, the hard palate, the saliva, the gingiva, the tongue, the throat and sub- or supragingival plaque. (Methé, Nelson et al. 2012)

The study was made with 242 healthy subjects leading to a total sample number of 4.788. The men were sampled at 15 different body sites, women at 18 (3 additional vaginal specimens). Because of the large sample size and sampling of many sites from the same individuals, this study gives an insight into the relationships between microbes. Researchers also draw conclusions about the influence of the microbes on different clinical parameters which could lead to understanding microbiome-based disorders. (Huttenhower, Gevers et al. 2012)

The findings reveal a great diversity of microorganisms in the human body, but also similarities between populations of different sites. The discovered species vary from one person to another. There seems to be a "core" microbiome – similar to the "core" genome that all human beings have in common. Further research thus tries to find relationships between the populations and possible reasons for irregularities or imbalance. Until further research is done, we can only assume that the composition of each individual's microbiome is influenced by several factors. For example environmental factors like diet or pharmaceutical exposures, as well as early life events and genetics. The microbiome may also play a role in our immune system. Some of the microorganisms that live on and in our bodies interact with both the innate and the acquired immune system.

1.2 The oral cavity

The oral cavity hosts hundreds of different species of bacteria. A large number of whom have not been cultivated before. (Aas, Paster et al. 2005) A study from 2001 detected 347 different species or phylotypes in subgingival plaque samples from healthy and diseased subjects. They estimate the total number of different species to be around 500. The nine bacterial phyla identified were: TM7, Obsidian Pool OB11, *Deferribacteres*, *Spirochaetes*, *Fusobacteria*, *Actinobacteria*, *Firmicutes*, *Proteobacteria* and *Bacteroidetes.* (Paster, Boches et al. 2001)

In 2010 the previous results were summarized as follows: "Approximately 280 bacterial species from the oral cavity have been isolated in culture and formally named. It has been estimated that less than half of the bacterial species present in the oral cavity can be cultivated using anaerobic microbiological methods and that there are likely 500 to 700 common oral species." (Dewhirst, Chen et al. 2010)

The oral cavity is constantly washed with fluid (saliva) that also contains nutritious macromolecules. There are areas that can host microaerophilic or facultative anaerobic microorganisms but also areas with no oxygen at all, where strict anaerobes grow best. Every time we eat or drink, nutrients enter the mouth and are cut down by enzymes in the saliva. This creates an ideal environment for bacteria. Some are even able to enzymatically digest proteins, which makes them independent from direct availability of carbohydrates. (Lindhe et al. 2008)

The mouth is a major gateway into the human body and an open system. Food is ingested, chewed, mixed with saliva before it enters the gastrointestinal tract. Air passes through mouth and nose before entering the lungs. Microorganisms from the environment are continually introduced through eating, drinking or breathing. It is therefore very difficult to differentiate between transient and endogenous bacterial species. Microorganisms entering the body via these ways can cause a number of infectious diseases, such as tonsillitis, caries or periodontitis. The latter two being caused by consortia of microorganisms, organized in so-called biofilms, rather than one single bacterium. (Dewhirst, Chen et al. 2010)

1.3 Biofilms

The formation of a biofilm takes place in several steps. First, some “pioneer” organisms adhere to a surface and then build a new surface for others. Often the produced metabolites are like a signal for other microorganisms to attach as well. This process is repeated several times until a biofilm is formed. (Jenkinson and Lamont 2005)

All biofilms consist of three components. The bacterial community forming the biofilm, the surface needed for attachment and the fluid passing over it, providing nutriments, flushing waste products or transporting cells. The nature of the surface on which a biofilm develops has an influence on its composition as shown in studies comparing sub- and supragingival plaque samples of the same individuals. (Socransky and Haffajee 2005)

The layer of macromolecules on non-shedding surfaces in the oral cavity is the origin of the biofilm. These strongly interconnected glycoproteins and microorganisms on the teeth are called plaque. Observation shows that the first plaque formers are Gram-positive facultative cocci and rods. The next microorganisms to adhere are then Gram-negative strictly anaerobic bacteria. Biofilms are complex, heterogeneous and present changing ecologic conditions. Most are formed by a dense layer of microorganisms bound together in a polysaccharide matrix together with other organic and inorganic materials in the lower levels. The top layers are looser and often irregular in appearance. The directly surrounding fluid layer is quite stationary, only layers above are in motion. This creates steep diffusion gradients, especially for oxygen. Biofilms form basically on every surface immersed in aqueous environments with a regular nutrient supply. They are ubiquitous. The specific structure makes the microorganisms resistant to antimicrobial agents. A biofilm can only be removed mechanically. (Lindhe et al. 2008)

Dental plaque was shown to be at the origin of gingivitis. A study made with periodontally healthy volunteers revealed development of gingivitis in all subjects after 10-21 days of withdrawal of toothbrushing. (Socransky and Haffajee 2005)

1.4 Microorganisms of the red complex

Studies showed that *P. gingivalis* tends to associate with other microorganisms, e.g. *Treponema denticola* or *Tannerella forsythia*. These three species belong to the so-called red complex. They preferably adhere to streptococci-rich plaque. And the virulence is higher than for the individual organisms alone. Other bacteria that have this effect in combination with *P. gingivalis* are: *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum*. The presence of red complex microorganisms increases the risk for periodontal diseases, especially for elderly people and smokers. (Jenkinson and Lamont 2005)

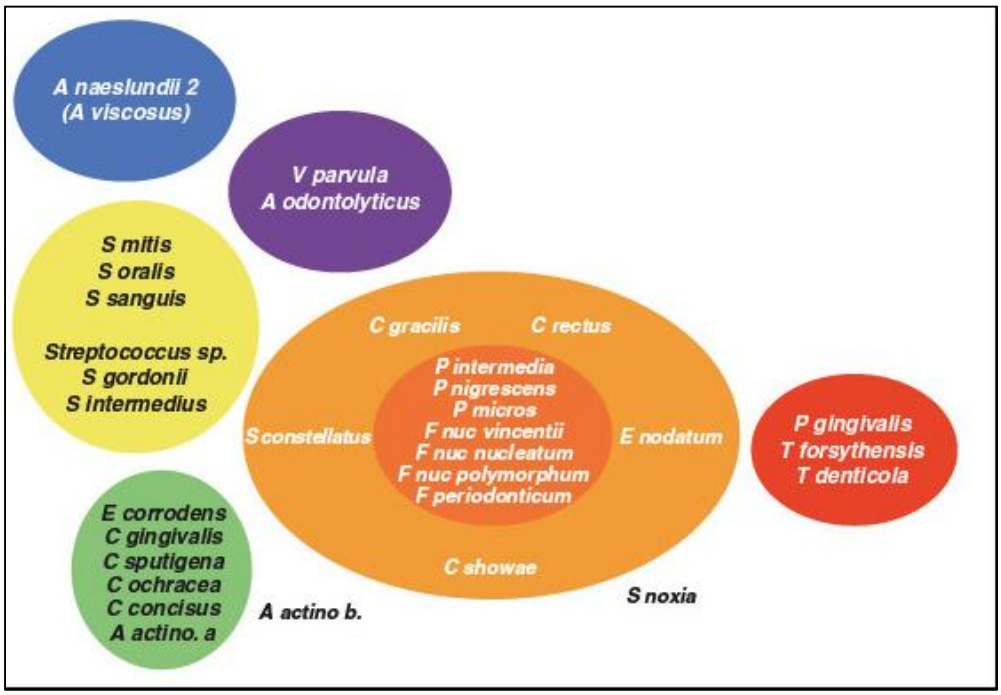

Figure 1: Microbial complexes in biofilms in the human oral cavity. Oxygen tolerance decreases from left to right. (Socransky, Haffajee et al. 1998)

1.4.1 *Porphyromonas gingivalis*

P. gingivalis presents coccal to short rod morphology and is a Gram-negative, non-motile, anaerobic and asaccharolytic microorganism. It is ranged in the group of the "black-pigmented *Bacteroides*" which play an important role in mixed infections and are much investigated. These organisms grow on blood agar and form brown to black colonies. *P. gingivalis* was discovered to produce a large range of virulence factors: collagenase, gingipain, different proteases (including those that destroy immunoglobulins), hemolysins, endotoxin, fatty acids, ammonia, hydrogen sulfide, indole, etc.

Studies have shown that the prevalence of *P. gingivalis* is significantly higher in subjects with destructive forms of periodontitis than in cases of health or gingivitis. They also induce elevated systemic and local immune responses in subjects with various forms of periodontitis. (Lindhe et al. 2008)

There is strong evidence that relates *P. gingivalis* to periodontitis. Several studies show that it is only rarely found in healthy subjects. On the other hand, there could be an underestimation of the actual occurrence of *P. gingivalis* due to low sensitivity of the detection methods. (Griffen, Becker et al. 1998)

Some studies support the concept that the occurrence of *P. gingivalis* is linked to the presence of other microorganisms. They are found to be more likely to adhere when the previous colonizers are streptococci. (Jenkinson and Lamont 2005)

1.4.2 *Tannerella forsythia* (formerly *Tannerella forsythensis* or *Bacteroides forsythus*)

T. forsythia is a Gram-negative, anaerobic, spindle-shaped, highly pleomorphic rod. It has been shown to produce trypsin-like proteolytic activity and methylglyoxal and induce apoptotic cell death. While at first thought to be a quite uncommon subgingival species, studies using monoclonal antibodies to examine plaque samples directly, revealed that it was present more abundantly than previously shown in cultural studies. Like *P. gingivalis*, *T. forsythia* is a periodontal pathogen rarely found in healthy subjects and more prevalent in subjects with various forms of periodontitis. (Lindhe et al. 2008)

T. forsythia was first described in the literature by Tanner et al. in 1979 after being isolated from the human oral cavity. It is an organism with very unique growth requirements, such as the iron-containing hemin, the vitamin K precursor menadione, the amino acid L-cysteine and *N*-acetylneuraminic acid and therefore difficult to grow. Its ability to induce apoptosis might play a role in the (rapid) progression of periodontitis. *P. gingivalis* and *T. forsythia* invade the periodontal pocket, induce an immunologic response and support the colonization by other pathogens through the elimination of the host immune or preimmune cells. (Dumitrescu 2010)

1.4.3 *Treponema denticola*

T. denticola is a Gram-negative, anaerobic, helical-shaped, highly motile microorganism that belongs to the spirochetes phylum. Spirochetes have been shown to be at the forefront of periodontal lesions

and more common in sub- than in supragingival plaque. *T. denticola* was more often found in patients with severe periodontitis than in healthy subjects or people with gingivitis. It can also be used as a marker for compliance with antibiotic treatment, since numbers significantly decrease in successfully treated periodontal sites but increase or do not change in non-responding sites. (Lindhe et al. 2008)

T. denticola is estimated to account for approximately 50% of the bacteria found in periodontal lesions and has the ability to penetrate the gingiva. It produces several virulence factors and has high proteolytic activity. Its capacity to decompose human matrix components facilitates the penetration and enhances also the invasion by other species. (Dumitrescu 2010)

Treponemes are difficult to isolate from healthy gingiva, but increase both in prevalence and number when the patient is affected by gingivitis or periodontal disease. A study from 1987 detected treponemes in 88-97% of subgingival plaque samples from patients with periodontal disease. The natural habitat of *T. denticola* is dental plaque in gingival crevices in the human oral cavity. (Murray et al. 2005)

1.5 Other periodontal pathogens

1.5.1 *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans is a small, non-motile, Gram-negative, saccharolytic, capnophilic, round-ended rod that forms small, convex colonies with a “star-shaped” center when grown on blood agar plates. It was recognized as a possible periodontal pathogen in the 1970s, when studies showed a higher prevalence in lesions of localized aggressive periodontitis compared to subjects with gingivitis or healthy subjects. *A. actinomycetemcomitans* produces a number of potentially damaging metabolites, including a leukotoxin and a cytolethal distending toxin. Studies have shown that it can invade gingival epithelial cells, buccal epithelial cells and vascular endothelial cells. It can also induce apoptotic cell death. Six serotypes (a, b, c, d, e, f) were detected so far. The suspected pathways of *A. actinomycetemcomitans* through the oral cavity are as follows: attachment to the surfaces of the oral epithelium, then to supragingival plaque or attachment to other colonizing bacterial species by coaggregation. At some point they are able to move to the subgingival environment and from then on possibly penetrate the underlying connective tissues. (Lindhe et al. 2008)

The species is microaerophilic and grows best in a humid atmosphere with 5-10% CO_2. It is commonly found in mucous membranes of the respiratory and genitourinary tracts of healthy individuals and develop their pathogenic potential when introduced into healthy (periodontal) tissue through lesions. *A. actinomycetemcomitans* is often detected in diseased tissue from adult patients affected by periodontal diseases and is also the most common cause for juvenile periodontitis. Detection of *A. actinomycetemcomitans* by cultural methods might be difficult due to overgrowth by other species present in diseased periodontal tissue samples. Molecular techniques such as PCR were therefore developed. (Murray et al. 2005)

1.5.2 *Campylobacter rectus*

C. rectus is a Gram-negative, anaerobic, short and motile vibrio. It requires hydrogen or formate as its energy source. Together with *A. actinomycetemcomitans* it is one of the two only known oral species that can produce a leukotoxin. *C. rectus* initiates periodontitis by stimulating the production of proinflammatory cytokines and is surrounded by a paracrystalline lattice (also called S-layer) that might enable it to survive in infected sites. It is widely distributed in subgingival sites (e.g. primary, mixed and permanent dentition of children) and more prevalent in diseased than in healthy sites. (Lindhe et al. 2008)

Campylobacters are non-spore-forming rods that can be curved, S-shaped or spiral. The motility is due to a single polar unsheathed flagellum on one or both ends. *C. rectus* has primarily been isolated from patients with periodontal diseases. (Murray et al. 2005)

1.5.3 *Eikenella corrodens*

E. corrodens is a Gram-negative, asaccharolytic, capnophilic, regular, small rod. It is facultatively anaerobic. The association of *E. corrodens* with periodontal disease is not as strong as for other pathogens mentioned in this work. Still, it is found frequently in actively breaking down periodontal sites and more often in patients responding poorly to treatment. (Lindhe et al. 2008)

Colonies of *E. corrodens* develop slowly, require 5-10% CO_2 and grow best on blood agar supplemented with hemin. It is commonly found in the oral cavity but also in the mucosa of the gastrointestinal and genitourinary tracts. In most infections, streptococci are also present. (Sheng, Hsueh et al. 2001)

E. corrodens has been detected in dental plaque, dental pockets, periodontal tissues, specimens from the lower respiratory tract, aspirates from wounds and abscesses, but also blood or other usually sterile fluids. It is responsible for both oral and extraoral infections. (Murray et al. 2005)

1.5.4 *Prevotella intermedia*

P. intermedia belongs to the *Bacteroides* genus and is a black-pigmented, Gram-negative, anaerobic, short and round-ended rod. Levels of this microorganism are shown to be elevated in patients with acute necrotizing ulcerative gingivitis, certain forms of periodontitis and progressing sites in chronic periodontitis. Studies with laboratory animals show that *P. intermedia* has a number of the virulence factors known from *P. gingivalis* and that it can induce mixed infections when injected. (Lindhe et al. 2008)

P. intermedia produces proteases and possesses various types of fimbriae. These fimbriae allow it to adhere to erythrocytes, leading to their agglutination. It was also related to bleeding on probing and, in co-presence of *T. forsythia*, to poorer response to treatment. (Dumitrescu 2010)

1.5.5 *Prevotella nigrescens*

P. nigrescens and *P. intermedia* are phenotypically identical. Bacteria of the genus *Prevotella* are among the first colonizers of the oral cavity of infants and are generally part of the indigenous microbiota of mucous membranes of humans and other mammals. Both *P. nigrescens* and *P. intermedia* are related to periodontitis, especially in the early onset of the disease when the periodontal pockets are not yet very deep. While *P. gingivalis* only develops in anaerobic sites, *P. nigrescens* and *P. intermedia* are more oxygen tolerant and can even create the necessary environment for further colonization by other anaerobes. Infectious processes associated with *P. nigrescens* include dental abscesses and endodontic and periapical infections. It is also known to produce volatile sulfur compounds and can therefore contribute to halitosis. Like *P. intermedia*, *P. nigrescens* produces several virulence factors, especially proteases. In fact, *P. intermedia* and *P. nigrescens* are the most proteolytic among all oral *Prevotella* species. (Liu 2011)

1.5.6 *Streptococcus mutans*

The genus *Streptococcus* belongs to the *Bacilli* class of microorganisms (phylum *Firmicutes*). They are the most abundant bacterial species to be found in the human oral cavity. (Dewhirst, Chen et al. 2010)

Streptococci are among the pioneer bacteria that first adhere to surfaces in the oral cavity and attract other microorganisms in order to form a biofilm. Initially, the bacteria bind to different saliva components and then to the surfaces. In the case of streptococci these saliva components are α-amylase, immunoglobulins, fibronectin, lactoferrin and α_2-macroglobulin. (Jenkinson and Lamont 2005)

Streptococci belong to the transient microflora of the skin and are resident colonizers of all kinds of mucous membranes (alimentary, respiratory and genital tracts). *S. mutans* is a Gram-positive, catalase-negative, facultatively anaerobic microorganism, forming spherical or ovoid cells. It is strongly related to dental caries. This is due to its ability to produce extracellular polysaccharides from sucrose and acid from a wide range of carbohydrates. Streptococci are oxygen-tolerant but preferably grow in a CO_2-enriched atmosphere. (Murray et al. 2005)

1.6 Rheumatoid arthritis

Rheumatoid arthritis is a chronic autoimmune disease with an intermittent course. Inflammatory processes occur in several areas of the body, mainly the synovial joints. It is also called chronic polyarthritis and diagnosed when five or more joints are affected. (Persson 2012) The consequences are not only the progressive destruction of the joints but also chronic pain, limited mobility and bone damage. Rheumatism is often considered as a “disease of old age”, but also young people or even children can be affected. Early diagnosis is very important in rheumatic diseases as the joints are irreversibly destroyed, even more so for the younger patients. For now, therapy consists in stopping the progression of the disease as the exact mechanisms are still unclear. When also treated with painkillers, most patients can lead a relatively “normal” life but of course the long-term aim of medical research is healing rheumatic diseases instead of practicing “damage control”.

Rheumatoid arthritis (RA) is the most common form of inflammatory diseases of the joints and often starts in the fingers or toes. Most patients show first symptoms between the age of 35 and 45 or after 60. Two thirds of the affected people are women. The reasons for this distribution are not

known yet. Besides the genetic disposition, one of the risk factors most researchers agree on is smoking. Tobacco abuse not only favours the initial development of the disease but also contributes to a faster progression. (Gesundheitsratgeber “Rheuma verstehen” 2012)

Besides the irreversible damaging of the joints, most likely due to the inflammatory processes, there are also systemic expressions of the disease. These include fatigue, fever, anemia, vessels and organ involvement. The production of two antibodies is characteristic for RA: rheumatoid factor (RF) and anti-citrullinated peptide antibody (ACPA). They act against common autoantigens expressed outside of the joints. Studies with monozygotic twins show an impact of genetic factors on the development of RA. But further research has to be done in order to fully understand the mechanisms involved. As environmental factors, that have an impact on the development of RA, most studies name tobacco abuse and the periodontal pathogen *Porphyromonas gingivalis*. (Boissier, Semerano et al. 2012)

1.7 Periodontitis

Periodontitis is a chronic disease initiated by an infection with certain microorganisms. The microorganisms that colonize the oral cavity are not completely identified yet. We probably know only about half of the possible causes for periodontal diseases today.

Chronic periodontitis often starts off as plaque-induced gingivitis, a reversible condition that becomes chronic if left untreated. Lesions related to chronic periodontitis are regarded as irreversible and include loss of attachment and bone.

Clinical features associated with the disease are:

- color, texture and volume alterations of the marginal gingiva
- recession of the gingival margin
- bleeding on probing from gingival pocket area
- increased pocket depth with reduced resistance of the soft marginal tissues to probing
- alveolar bone loss
- root furcation exposure
- increased tooth mobility
- drifting and eventually exfoliation of teeth

Further characteristics of chronic periodontitis are a higher prevalence in adults than in children, the association with oral hygiene and plaque levels, as well as environmental factors such as smoking,

stress and systemic risk factors. The bacterial composition of the subgingival biofilm is highly variable – not only between diferent subjects but also on different sites in the same subject. Host factors determine the severity of the infection and the speed of progression. (Lindhe et al 2008)

Several studies relate cigarette smoking to periodontal diseases. Smokers tend to have deeper periodontal pockets, more alveolar bone loss and greater attachment level loss. When looking at the different species identified in plaque samples, there was often no significant difference between smokers and non-smokers. However, the prevalence of some species of the orange complex and all species of the red complex was shown to be significantly higher in smokers. (Socransky and Haffajee 2005)

Obesity is also suspected to play a role in the development of periodontitis. Comparable to the findings on smokers, it was shown that subjects with an elevated BMI have significantly deeper periodontal pockets, show more bleeding on probing and sites with plaque than non-obese subjects. (Socransky and Haffajee 2005)

Genetic factors and geographic location also play a role in susceptibility for periodontitis, but the mechanisms and reasons are not fully explained yet. Genes define the proteins present in a person's saliva – which is the bulk fluid of the biofilm in the oral cavity and therefore defines the species present and their relative amounts. The geographic location on the other hand might have an impact through socioeconomic factors: lifestyle, dietary habits, oral hygiene, etc. (Socransky and Haffajee 2005)

1.8 Selection of methods

1.8.1 Detection and enumeration of bacteria in oral biofilm samples

Detection and enumeration is a very challenging task when analyzing oral biofilm samples. First of all, the samples contain a very high number of different microorganisms since the oral cavity is densely populated. Additionally, most of the bacterial species present in oral biofilm samples are fastidious organisms and not easy to cultivate. (Lindhe et al. 2008)

This fact played an important role in the choice of methods and materials. Microbiological methods require a lot of expertise – both before and after the cultivation period. It is necessary to know what nutrient media are best, what growth conditions are required and then how to differentiate between the big number of species present in the samples.

In order to differentiate between periodontal health and disease, quantification is necessary. (Lindhe et al. 2008) At least the relative amounts of the different microorganisms should be detected in order to make assumptions about the composition of the oral biofilm.

Culture-independent techniques like the 16S rRNA gene-based molecular cloning methods have widely replaced “classical” cultivation, since it allows the detection of microorganism that have not been cultivated before. (Dewhirst, Chen et al. 2010)

1.8.2 Cultural techniques

Cultural techniques are specific and allow the distinction of several species, but also very expensive. (Lindhe et al. 2008) This means either to generate a large budget or to limit the number of samples.

Estimates of bacterial count through cultivation lack accuracy for total bacteria, since many of the species present in the oral cavity and periodontal pockets are difficult to culture or haven’t been cultured at all so far. Additionally, for specific species, e.g. *Porphyromonas gingivalis*, the sensitivity is lower. (Lyons, Griffen et al. 2000)

Some oral pathogens show extremely slow growth or have very specific growth requirements. Cultural techniques can therefore become laborious, time consuming and show a lack of sensitivity. (Boutaga, Van Winkelhoff et al. 2003)

Although cultivation is considered as golden standard, there are limitations: some periodontal pathogens do not show reliable growth on selective media and non-viable microorganisms will not be detected. (Jervøe-Storm, Koltzscher et al. 2005)

1.8.3 Immunoassays

By definition, immunoassays are procedures that detect antibodies or antigens in patients in order to detect whether they are infected and/or present an immunologic response to an infection. There are two general approaches: testing for specific microbial antigens or for microbial antigen-specific antibodies. Immunoassays can be designed as liquid-phase or solid-phase assays. As reaction labels a radioimmunoassay uses a radioactive label, an enzyme immunoassay uses enzymes that react with a certain substrate and a fluorescence immunoassay uses fluorescent dyes. (Murray et al. 2005)

The surfaces used for solid-phase immunoassays can be made of plastic, glass, polyvinyl chloride (PVC), nitrocellulose, cellulose, agarose, polyacrylamide or dextran. In order to avoid overload or

unwished protein-protein interactions, the washing steps in the protocol have to be respected strictly. (Murray et al. 2005)

Enzyme immunoassays use either an antibody or an antigen labelled with an enzyme which ideally has a high turnover rate, conjugates easily, is not endogenously present in the patient sample, easy to detect, compatible with the standard conditions used, stable and not expensive. The corresponding substrate can be radioactive, fluorescent, chromogenic or chemiluminescent. (Murray 2005)

Immunofluorescence or enzyme-linked immunosorbent assays (ELISA) can be used for the analysis of oral biofilms. The specificity of these methods is high and they allow quantification. But they are limited to a certain number of species of which antisera were developed. Samples may therefore not be fully analyzed when using this method. (Lindhe et al. 2008)

1.8.4 Molecular techniques

Molecular techniques such as PCR or DNA hybridization allow the detection of a wide range of bacteria and are highly specific. If compared to one another, the advantages of some techniques over others are clear. PCR is a well-known technique and allows the detection of low cell numbers, but no quantification. Real-time PCR on the other hand gives information about the quantity of microorganisms present in the sample but is rather expensive and time-consuming. A quite convenient way to analyse large numbers of samples without getting too expensive is DNA hybridization. The technique is sensitive, specific and allows high throughput. Provided the necessary tools and machines are available, this method seems to be the most useful at the moment. (Lindhe et al. 2008)

PCR detection shows higher specificity and sensitivity than cultural methods: based on gene-specific DNA sequences it allows the distinction of closely related bacteria. (Jervøe-Storm, Koltzscher et al. 2005)

Real-time PCR is a reliable and efficient quantification method that offers high sensitivity. With species-specific primers it is a precise technique for accurate quantitation of individual species. (Lyons, Griffen et al. 2000)

The detection of unexpected pathogens could be interesting for a general analysis of the pathogenic load of a sample. This possibility is ruled out by the use of species-specific primers. (Mackay 2007)

Since this work aims to detect nine selected microorganisms, the use of less specific detection methods is not requested.

Polymerase Chain Reaction (PCR)

The biochemist Kary Mullis developed this method in the beginning of the 1980s. It allows the replication of genes through the use of so-called primers. These are oligonucleotides of generally 20-25 bases in length, whose sequence is complementary to parts of the fragment to be replicated. They anneal to the single-stranded DNA and mark the starting point of the DNA synthesis. Further necessary elements are: DNA polymerase and nucleotides. (Wink 2011)

Generally the PCR has 3 stages: denaturation, annealing and extension. At 94-96°C the double-stranded DNA is denatured into single strands. The primers then anneal to the complementary sequence at a temperature close to the melting temperature, usually between 40 and 60°C. The extension step involves the DNA polymerase which uses the nucleotides in the reaction mixture to elongate the oligonucleotide primers. It is derived from *Thermus aquaticus*, a heat-stable bacterium with an optimum activity at a temperature of 72°C. The polymerase is not destroyed during the denaturation step and therefore has not to be freshly added throughout the reaction. (Wink 2011)

The three steps are repeated in so-called cycles (20 to 40 times), which theoretically lead to an exponential replication of the given DNA template. In reality the activity of the polymerase decreases in later cycles, as well as the quantity of primers and nucleotides. (Wink 2011)

There are also protocols which consist in only two steps. Due to the broad thermal range of activity of the polymerase of 70-75°C, it is possible to combine the annealing and extension step. (Mackay 2007)

The melting temperature (T_m).is the temperature at which 50% of the nucleotides remain hybridized with the target sequence. It depends on the sequence of the oligonucleotide, its length, the concentration of the DNA and the composition of the solvent in which the DNA is suspended. Usually the annealing temperature used for the PCR reaction is 5-10°C below the T_m. (Mackay 2007)

After the amplification of the target sequence by PCR, the detection is done by agarose gel electrophoresis. Ethidium bromide binds to the DNA and is then visualized by irradiation with ultraviolet light. (Mackay 2007)

Real-time PCR

Real-time PCR has big advantages when it comes to choosing the best method for microbial detection: high sensitivity, specificity and speed. The main difference to conventional PCR is the elimination of the detection step by agarose gel electrophoresis which saves much time.

Amplification and detection are combined in one single step and the whole process can therefore take place in the same closed reaction vessel. There are no post-PCR manual manipulations needed and the risk for variability or contamination is widely eliminated. (Edwards et al. 2004)

Real-time PCR Thermocyclers

A programmable thermal cycler controls the temperature, time and length of each cycle. The reaction runs automatically after being programmed with the corresponding software. Some cyclers can reduce the time needed for an assay even more by the use of electronically controlled heating blocks or fan-forced heated airflows. (Mackay 2007)

Thermocyclers can be Peltier-based: the temperature changes are induced by heating blocks in which the reaction vessels are standing. Other heat exchange technologies are: a stationary air-heated glass capillary format ("LightCycler"), a high-thermal-conductivity ceramic heating plate plastic tube format ("SmartCycler") and a centrifugal air-heated plastic tube format ("RotorGene"). The "RotorGene" thermocycler allows shorter run times than the others and shows the smallest variations in temperature uniformity. (Edwards et al. 2004)

Real-time PCR Detection

Unlike the detection by agarose gel electrophoresis for conventional PCR, the detection for real-time PCR happens simultaneously to the reaction. The principle is the same though: detection via fluorescent dyes. Ethidium bromide is no longer used, but was replaced by several other substances. There are two types: the ones interacting with any and all double-stranded DNA (e.g. SYBR® Green I) and sequence-specific fluorogenic oligoprobes (e.g. TaqMan® oligoprobes). In general both methods detect amplicons with similar sensitivity, the advantage of the non-specific dyes is that they are minimally affected by small changes in the template's sequence. When free in solution these substances show minimal fluorescence but emit strongly when associated to the minor groove of the DNA duplex and exposed to a certain wavelength of light. (Mackay 2007)

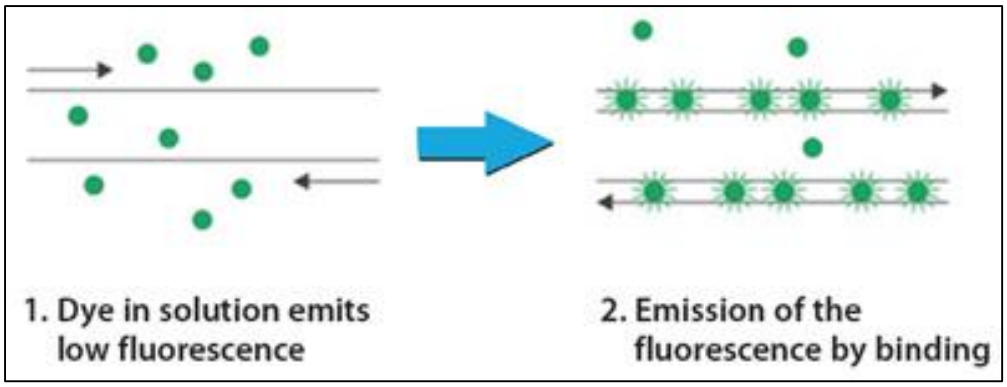

Figure 2: The principle of the fluorescent dye SYBR® green. Fluorescence emission is high when bound to double-stranded DNA. (www.sigmaaldrich.com)

Besides SYBR ® Green I, there is another quite popular dye: SYBR® Gold. Both dyes are commonly used because their emission maxima are in the range of most commercial real-time PCR instruments (around 520 nm). They usually exhibit 20-100 fold fluorescence enhancement on binding to double-stranded DNA (dsDNA). The use of the SYBR® dyes is quite easy, there is no need for additional experimental design and many manufacturers offer compatible reagents. (Edwards et al. 2004)

Real-time PCR Problems

A common problem during PCR is primer-dimer formation. It occurs during the plateau phase of amplification and can lead to a misinterpretation of the results as the DNA-binding fluorophores can also associate with primer-dimers (or any non-specific amplification product). When analyzing purified DNA from patient samples for the presence of microbial targets, the number of templates is often low. Unfortunately this favours the formation of primer-dimers. The best way to reveal this, is to use software capable of melting curve analysis producing fluorescent dissociation curves. (Mackay 2007)

To some extent the melting curve analysis allows to differentiate between signals given by specific amplicons and non-specific artefacts. Generally the specific reaction products melt at higher temperatures than for example primer-dimers or mis-primed PCR products. (Edwards et al. 2004)

Real-time PCR Results

For an optimal reaction it is important to use the right amounts of the different components. Too little polymerase for example leads to inefficient amplification and low fluorescence, which means loss of sensitivity. Too much of it also leads to low fluorescence and can contribute to the formation

of primer-dimers or other non-specific amplicons. In case of the dye, SYBR ® Green I, too high concentrations can inhibit the Taq polymerase and therefore adversely affect the reaction. (Logan et al. 2009)

It is also important to consider the aim of the work before starting it. The type of assay applied depends on the problem to solve. There is no use in performing a quantitative detection if only qualitative information is required. It is also important to adapt the cycling conditions, if they were designed for regular PCR and are now applied in a real-time assay. (Logan et al. 2009)

Real-time PCR Melting Curve Analysis

Real-time PCR instruments come with the right software for data analysis. There are three different types, the one used in this work is observation and analysis of the so-called melting curves. Fluorescence measurements are made while the temperature is slowly increasing from around 50°C up to around 95°C. The fluorescence decreases as the bound dye is released because the duplexes melt apart. Through plotting –dF/dT against T (where F=fluorescence and T=temperature) we obtain one or more melting peaks that indicate the maximum rate(s) of change in fluorescence. These are characteristic for specific dsDNA products. The melting temperature depends on the GC content and the length of the fragment which allows identification. (Edwards et al. 2004)

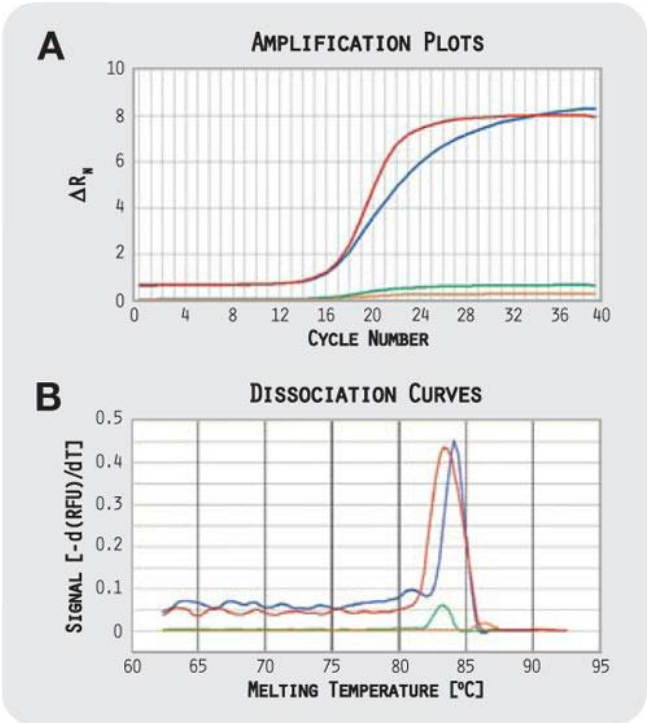

Figure 3: A: Fluorescence emission rises during the PCR reaction. B: The peak shows the melting temperature. (www.sabiosciences.com)

2 Scope

The aim of this work was to investigate the microbiome of the human oral cavity in order to find possible connections between periodontitis and rheumatoid arthritis. It also aimed to find out if there were dietary influences on the microorganisms that colonize the oral cavity. The topic was approached by first finding an appropriate method to detect specific periodontal pathogens in human plaque samples.

3 Material & Methods

3.1 Material

- Agar, bacteriologic (Agar no. 1)

LP 0011; Lot 1265897-02

Oxoid Ltd; Basingstoke, Hampshire, England

- Agarose: Biozym LE Agarose

Art.-Nr. 840004; Lot No. 120212C276589L

Biozym Biotech Trading GmbH; Wien, Austria

- Autoclave: CertoClav®

CertoClav Sterilizer GmbH; Traun, Austria

- Blood: sterile defibrinated sheep blood

Oxoid Ltd; Basingstoke, Hampshire, England

- Brain Heart Infusion agar

CMG 1136; Lot 1352225

Oxoid Ltd; Basingstoke, Hampshire, England

- Brain Heart Infusion broth

CM 1135; Lot 1352767

Oxoid Ltd; Basingstoke, Hampshire, England

- Centrifuge 5417 R

Serial No. 5407YK628724

Eppendorf AG; Hamburg, Germany

- Chocolate agar PolyViteX

BioMérieux SA; Lyon, France

- L-Cysteine-HCl hydrate, 99%

Cat.: C12, 180-0; Lot 09209KC-424

Sigma-Aldrich Chemie GmbH; Steinheim, Germany

- DNA extraction kit: peqGOLD Bacterial DNA kit

Order No. 12-3450-02; Lot 040813

Peqlab; Erlangen, Germany

- DNA ladder 100 bp

AppliChem GmbH; Darmstadt, Germany

- Ethidium bromide solution

Art. Nr. 2218.2; Charge 261173611

Carl Roth GmbH + Co KG; Karlsruhe, Germany

- Glycerine

Art. Nr. 7533.1; Charge 38466707

Carl Roth GmbH + Co KG; Karlsruhe, Germany

- Lab-Lemco powder

LP 0029; Lot 334312

Oxoid Ltd; Basingstoke, Hampshire, England

- Master Mix: Rotor-Gene™ SYBR® Green PCR Master Mix (2x)

Mat. No. 1063613; Lot No. 142343186

Qiagen; Hilden, Germany

- Nutrient agar

Art. Nr. X928.1; Charge 190152241

Carl Roth GmbH + Co KG; Karlsruhe, Germany

- Peptone, neutralised bacteriological

LP 0034; Lot 1199626

Oxoid Ltd; Basingstoke, Hampshire, England

- Real-time PCR device: Rotor-Gene Q

Serial No. R1111142

Qiagen; Hilden, Germany

- Shaker: Thermomixer comfort

No. 535536770

Eppendorf AG; Hamburg, Germany

- Sodium chloride

Art. Nr. 3957.1; Charge 468101183

Carl Roth GmbH + Co KG; Karlsruhe, Germany

- Submerged Gel Electrophoresis Apparatus SEA 2000®

Serial No. 98-GRI-831

Elchrom Scientific AG; Switzerland

- TE buffer (pH 7,85)

0,2 ml EDTA (0,5M) + 1 ml TRIS-HCl (1M) in 100 ml HPLC-water; autoclaved for 15 min at 121°C, 2 bar

- TAE buffer (0,75x)

15 ml TAE buffer (50x) in 1000 ml UHQ-water

- Transilluminator: BIO-RAD Molecular Imager® Gel Doc™ XR+ with Image Lab™ Software

Model No. Universal Hood II; Serial No. 721BRO4272

Bio-Rad Laboratories Inc.; Hercules, CA, USA

- Wilkins-Chalgren anaerobe broth

CM 0643; Lot 857949

Oxoid Ltd; Basingstoke, Hampshire, England

3.2 Work procedures

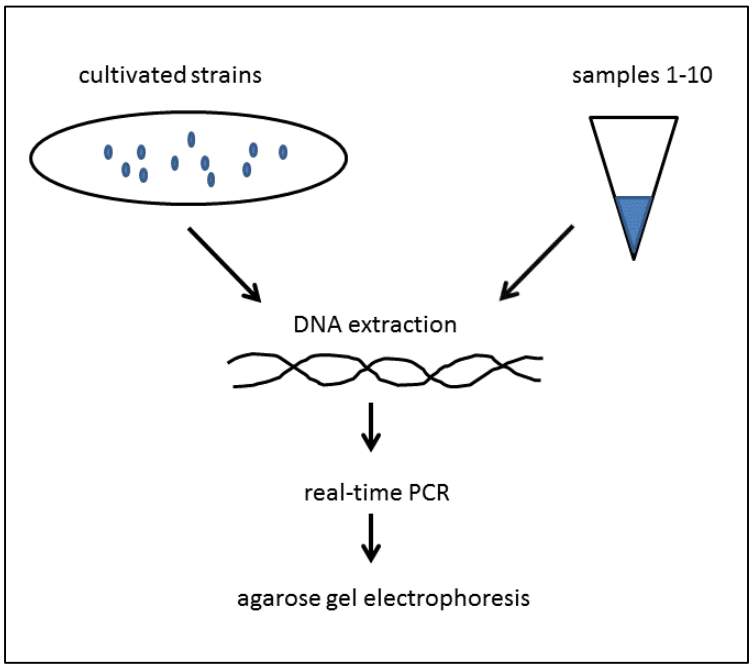

Figure 4: The cultivated strains serve as positive control for the analysis of the samples. They are also used to test the primer systems on specificity and cross-reactions.

3.3 Media preparation

3.3.1 Sodium Chloride solution 0,9% (NaCl)

0,9 g sodium chloride in 100 ml RO-water; autoclaved for 15 min at 121°C, 2 bar

3.3.2 Blood agar plates

Plates were made according to the recommendations given by the "Laboratorium voor Microbiologie, Universiteit Gent" (BCCM/LMG) for the cultivation of *E. corrodens*.

Mix 10 g/l Lab-Lemco, 10 g/l peptone, 5 g/l NaCl and 15 g/l agar. Add distilled water. Autoclave and aseptically add 50 ml sterile blood per liter of molten medium at 45°C.

The agar was then poured into disposable sterile petri dishes and after solidification the plates were stored in a portable anaerobic system at 4°C.

3.3.3 Nutrient agar plates

Plates were made according to the recommendations given by the "Laboratorium voor Microbiologie, Universiteit Gent" (BCCM/LMG) for the cultivation of *C. rectus*.

Prepare nutrient agar according to the manufacturer's instructions. Add 2 g/l Na-formate and 3 g/l fumaric acid. Adjust pH to 7,2 by adding KOH. After autoclaving, aseptically add 50 ml of sterile blood per liter of molten medium at 45°C.

The agar was then poured into disposable sterile petri dishes and after solidification the plates were stored in a portable anaerobic system at 4°C.

3.4 Cultivation

3.4.1 *Campylobacter rectus*

A living culture of the type strain (strain nr. LMG 7615) was provided on an agar plate by the "Laboratorium voor Microbiologie, Universiteit Gent" (BCCM/LMG).

The cells were harvested by suspending the colonies in 2 ml of Brain Heart Infusion (BHI) with L-cysteine hydrogen chloride (0,5 g/l) with a disposable spatula. For further cultivation the suspension was added to test tubes containing the same liquid medium and used to inoculate nutrient agar plates.

All cultures were incubated under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) at 37°C for 12 days.

For further cultivation, eight more plates of nutrient agar were inoculated with 0,1 ml each, out of one of the liquid culture tubes. Incubation as described above for 9 days.

The colonies from the previously incubated plates were harvested by pipetting 1 ml of NaCl on the agar surface and suspending the cells in the medium with a disposable spatula. The suspension was pipetted into Eppendorf tubes and centrifuged (8000 rpm, 10 min). The supernatant was nearly completely discarded and the procedure repeated. The pellets were then stored at -30°C.

The liquid cultures were used to inoculate more test tubes containing BHI medium by transferring 1 ml of the previous cultures into a test tube with 5 ml of fresh medium.

After 7 days of cultivation, the liquid cultures were transferred into Eppendorf tubes in 2 ml aliquots. The tubes were centrifuged at 8000 rpm for 6 min, washed with NaCl once and centrifuged once more. The supernatant was almost completely removed and discarded. The pellets were then stored in the Eppendorf tubes at -30°C.

3.4.2 *Eikenella corrodens*

A freeze dried culture of the type strain (strain nr. LMG 15557) was provided by the "Laboratorium voor Microbiologie, Universiteit Gent" (BCCM/LMG).

After breaking the glass vial open, the pellet was resuspended in Brain Heart Infusion (BHI) with L-cysteine hydrogen chloride (0,5 g/l). The suspension was then used to inoculate two test tubes containing the same liquid medium and two blood agar plates.
All cultures were incubated at 37°C for 12 days.

The cells from the agar plates were then harvested by flushing with 1 ml of NaCl twice and pipetting the suspension into Eppendorf tubes. After centrifugation at 8000 rpm for 6 min, the pellets were washed with NaCl once and then stored at -30°C.

The liquid cultures were used to inoculate more test tubes containing BHI medium by transferring 1 ml of each tube from the previous cultures into test tubes containing 5 ml of fresh medium each.

After 7 days of incubation, the liquid cultures were transferred into Eppendorf tubes in 2 ml aliquots. The tubes were centrifuged at 8000 rpm for 6 min, washed with NaCl once and centrifuged once more. The supernatant was almost completely removed and discarded. The pellets were then stored in the Eppendorf tubes at -30°C.

3.4.3 *Porphyromonas gingivalis*

A living culture of the type strain (strain nr. DSM 20709) was provided by the "Deutsche Sammlung für Mikroorganismen und Zellkulturen" (DSMZ).

For further cultivation, Wilkins-Chalgren anaerobe broth was made according to the manufacturer's instructions. The medium was then autoclaved in test tubes containing 10 ml aliquots. Each test tube was inoculated with 10 drops of the provided living liquid culture and incubated at 37°C for 14 days under anaerobic conditions.

The liquid cultures were then transferred into Eppendorf tubes (2 ml, safe lock) in 2 mL aliquots. The cells were washed once with NaCl and partly directly stored at -30°C or mixed with 200 µl of glycerine and stored at -80°C.

3.4.4 *Streptococcus mutans*

A freeze dried culture of the type strain (strain nr. LMG 14558) was provided by the "Laboratorium voor Microbiologie, Universiteit Gent" (BCCM/LMG).

After breaking the glass vial open, the pellet was resuspended in Brain Heart Infusion (BHI) with cysteine-HCl (0,5 g/l). The suspension was then used to inoculate two test tubes containing the same liquid medium and two BHI agar plates.

All cultures were incubated under anaerobic conditions at 37°C for 12 days.

The cells from the agar plates were then harvested by flushing with 1 ml of NaCl twice and pipetting the suspension into Eppendorf tubes. After centrifugation at 8000 rpm for 6 min the pellets were washed with NaCl once and then stored at -30°C.

The liquid cultures were used to inoculate more test tubes containing BHI medium by transferring 1 ml of each of the tubes from the previous cultures into test tubes containing 5 ml of fresh medium each.

After 7 days of incubation, the liquid cultures were transferred into Eppendorf tubes in 2 ml aliquots. The tubes were centrifuged at 8000 rpm for 6 min, washed with NaCl once and centrifuged once more. The supernatant was almost completely removed and discarded. The pellets were then stored in the Eppendorf tubes at -30°C.

3.4.5 *Tannerella forsythia* (formerly *Tannerella forsythensis* or *Bacteroides forsythus*)

A freeze dried culture of the type strain (strain nr. CCUG 21028A) was provided by the "Culture Collection, University of Göteborg, Sweden" (CCUG).

After breaking the glass vial open, the pellet was resuspended in 1 ml of Wilkins-Chalgren anaerobe broth, made according to the manufacturer's instructions. The suspension was then transferred into a test tube containing 10 ml of Wilkins-Chalgren anaerobe broth and incubated at 37°C for 7 days under anaerobic conditions.

For further selective cultivation two plates of Chocolate agar were inoculated with 0,1 ml each and incubated under the conditions described above. After 7 more days, 10 more Chocolate agar plates were inoculated with the liquid culture from the test tube and the cells from the first two plates were harvested.

Harvesting consisted in suspending the colonies in 2 ml of NaCl with a disposable spatula and transferring the suspension into Eppendorf tubes (2 ml, safe lock). The cells were washed once with NaCl, pelleted and stored at -30°C.

After 16 days of incubation, the cells from the 10 additional plates were harvested in the same manner and the pellets were stored in Eppendorf tubes at -30°C.

Six more plates of Chocolate agar were inoculated with 0,1 ml of the liquid culture and incubated under anaerobic conditions for 14 days at 37°C. The cells were harvested as previously described and the pellets stored in Eppendorf tubes at -30°C.

The remaining liquid culture was transferred into Eppendorf tubes in 2 ml aliquots. The tubes were centrifuged at 8000 rpm for 10 min, washed with NaCl once and centrifuged once more. The supernatant was almost completely removed and discarded. The pellets were then stored in the Eppendorf tubes at -30°C.

3.4.6 Other strains

Type strains of *Aggregatibacter actinomycetemcomitans*, *Prevotella nigrescens*, *Prevotella intermedia* and *Treponema denticola* were not available or too difficult to cultivate. The DNA was therefore provided by the "Deutsche Sammlung für Mikroorganismen und Zellkulturen" (DSMZ).

All DNAs (volume V=25 µl) were stored in TE buffer (10 mM Tris pH 7,5; 1 mM EDTA) at -30°C.

The concentrations were as follows:

Table 1: Concentrations of the DNAs delivered by the DSMZ.

Species name	DSM number	Concentration [ng/µl]
Aggregatibacter actinomycetemcomitans	DSM 8324	117,7
Prevotella nigrescens	DSM 13386	271,3
Prevotella intermedia	DSM 14222	n.a.
Treponema denticola	DSM 20706	n.a.

3.5 Sampling

All participants received the following material:

- 1 questionnaire
- 1 instruction sheet for sampling
- 1 Eppendorf tube containing 0,5 ml TE buffer (pH 7,85)
- 1 plastic toothpick

3.5.1 Questionnaires

The questionnaires consisted of three parts. At first, 28 questions concerning age, gender, health status, education and general lifestyle (working environment, smoking, etc.). The second part was a food frequency questionnaire: a large range of foodstuffs and drinks is listed and the participants state their average consumption of each one. The categories were: (almost) never; 1-3 times a month; once a week, 2-3 times a week, 4-6 times a week; once a day, 2-3 times a day, 4 times a day or more. The third part was a 24h-recall: what did each participant eat and drink during the last 24 hours?

Due to the low number of participants (10), the questionnaires will mainly be analysed concerning their practicability and comprehensiveness. Statistical analysis would only be interesting with more data.

3.5.2 Sampling instructions

The participants were instructed to scratch closely to the gingiva over one or more teeth with the tip of the plastic toothpick. They were also instructed to scratch between teeth, if possible. The thus obtained sample should be transferred into the buffer in the Eppendorf tube by spinning the toothpick inside the liquid. Furthermore, the participants were asked to not hand in bloody samples and not to take the samples immediately after eating or toothbrushing.

3.5.3 Sample handling

The Eppendorf tubes were stored refrigerated until further processing. The DNA extraction was made with a commercially available kit: "peqGOLD Bacterial DNA Kit" (peqlab, Germany). The samples were treated according to the instructions in the enclosed manual.

Varying conditions:

Step 1: Cell lysis for Gram-positive bacteria: 100 µl TE buffer + 100 µl lysozyme

Step 6: Elution buffer volume: 100 µl

The extracted DNA was stored in Eppendorf tubes (1,5 ml; safe lock) at -30°C.

3.6 Primers

3.6.1 Primer selection

The specific primers were designed according to different sources and ordered at Eurofins MWG Operon (Ebersberg, Germany):

According to A. Ashimoto et al (1996):

Table 2: Target organism, abbreviation, sequence and amplicon length of the specific primer pairs selected from (Ashimoto, Chen et al. 1996).

Microorganism	Type strain nr.	Primer pair	Sequence	Amplicon length
A. actinomycetemcomitans	DSM 8324	Aa557F	5'-AAA CCC ATC TCT GAG TTC TTC TTC-3'	557 bp
		Aa557R	5'-ATG CCA ACT TGA CGT TAA AT-3'	
C. rectus	LMG 7615	Cr598F	5'-TTT CGG AGC GTA AAC TCC TTT TC-3'	598 bp
		Cr598R	5'-TTT CTG CAA GCA GAC ACT CTT-3'	
E. corrodens	LMG 15557	Ec688F	5'-CTA ATA CCG CAT ACG TCC TAA G-3'	688 bp
		Ec688R	5'-CTA CTA AGC AAT CAA GTT GCC C-3'	
P. intermedia	DSM 20706	Pi575F	5'-TTT GTT GGG GAG TAA AGC GGG-3'	575 bp
		Pi575R	5'-TCA ACA TCT CTG TAT CCT GCG T-3'	
P.nigrescens	DSM 13386	Pn804F	5'-ATG AAA CAA AGG TTT TCC GGT AAG-3'	804 bp
		Pn804R	5'-CCC ACG TCT CTG TGG GCT GCG A-3'	
T. denticola	DSM 14222	Td316F	5'-TAA TAC CGA ATG TGC TCA TTT ACA T-3'	316 bp
		Td316R	5'-TCA AAG AAG CAT TCC CTC TTC TTC TTA-3'	
T. forsythia	CCUG 21028	Bf641F	5'-GCG TAT GTA ACC TGC CCG CA-3'	641 bp
		Bf641R	5'-TGC TTC AGT GTC AGT TAT ACC T-3'	

According to V. Zijnge et al (2006):

Table 3: Target organism, abbreviation, sequence and amplicon length of the specific primer pair selected from (Zijnge, Welling et al. 2006).

Microorganism	Type strain nr.	Primer pair	Sequence	Amplicon length
P. gingivalis	DSM 20709	Pg1132	5'-ACT GTT AGC AAC TAC CGA TGT-3'	404 bp
		Pg729	5'-AGG CAG CTT GCC ATA CTG CG-3'	

According to E. Kozarov et al (2006):

Table 4: Target organism, abbreviation, sequence and amplicon length of the specific primer pairs selected from (Kozarov, Sweier et al. 2006).

Microorganism	Type strain nr.	Primer pair	Sequence	Amplicon length
A. actinomycetemcomitans	DSM 8324	Aa276F	5'-GGC ACG TAG GCG GAC CTT-3'	276 bp
		Aa276R	5'-ACC AGG GCT AAA GCC CAA TC-3'	
E. corrodens	LMG 15557	Ec58F	5'-GGG AAG AAA AGG GAA GTG CT-3'	58 bp
		Ec58R	5'-TCT TCA GGT ACC GTC AGC AAA A-3'	
P. gingivalis	DSM 20709	Pg437F	5'-CAT AGA TAT CAC GAG GAA CTC CGA TT-3'	437 bp
		Pg437R	5'-AAA CTG TTA GCA ACT ACC GAT GTG G-3'	
P. intermedia	DSM 20706	Pi86F	5'-AGA TTG ACG GCC CTA TGG GT-3'	86 bp
		Pi86R	5'-CCG GTC CTT ATT CGA AGG GTA-3'	
S. mutans	LMG 14558	Sm90F	5'-AGC GTT GTC CGG ATT TAT TGG-3'	90 bp
		Sm90R	5'-AGA GCA CAC TAT GGT TGA GCC A-3'	
T. denticola	DSM 14222	Td63F	5'-CTT CCG CAA TGG ACG AAA GT-3'	63 bp
		Td63R	5'-CAA CCT TTC GGC CTT CTT CA-3'	
T. forsythia	CCUG 21028	Tf127F	5'-GGG TGA GTA ACG CGT ATG TAA CCT-3'	127 bp
		Tf127R	5'-CAA CCT TTC GGC CTT CTT CA-3'	

According to W. Psoter et al (2011):

Table 5: Target organism, abbreviation, sequence and amplicon length of the specific primer pair selected from (Psoter, Ge et al. 2011)

Microorganism	Type strain nr.	Primer pair	Sequence	Amplicon length
S. mutans	LMG 14558	Sm479F	5'-TCG CGA AAA AGA TAA ACA AAC A-3'	479 bp
		Sm479R	5'-GCC CCT TCA CAG TTG GTT AG-3'	

All primers were used in a 10 pmol/µl concentration.

3.6.2 Primer testing

In order to test the primers for their specificity, a DNA mix was made, containing DNA of all microorganisms that should be detected.

2 µl of DNA of each species were mixed in an Eppendorf tube (0,5 ml; safe lock), diluted 1:1 with RO-water and stored at -30°C.

After amplification with real-time PCR under the same conditions as the sample analysis, the PCR products were checked by agarose gel electrophoresis. There should be an amplification product of the expected size for every primer used and no unspecific bands.

3.7 Real-time PCR

3.7.1 Reaction parameters

Each tube contained a reaction volume of 20µl:

Master-Mix	10 µl
Specific forward primer	0,5 µl
Specific reverse primer	0,5 µl
UHQ-H_2O	8 µl
DNA	1 µl

The chosen parameters were:

95°C	5 min	activation of the Taq polymerase	
95°C	30 sec	denaturation step	35 cycles
60°C	60 sec	annealing/extension step	

Melting curve analysis:

Temperature range was chosen between 78-93°C. Except for the assay Ec 58, where it was 68-85°C. The temperature changed in 0,1 degree steps.

3.7.2 Positive controls

Each assay was made in duplicate and included a negative and a positive control. While the negative control was made with UHQ-water instead of DNA, the positive control contained 1 µl of the DNA of the species tested for.

For the cultivated strains *C. rectus*, *E. corrodens*, *P. gingivalis*, *S. mutans* and *T. forsythia*, DNA extraction was made with a commercially available kit: "peqGOLD Bacterial DNA Kit" (peqlab, Germany). For each strain, one tube containing the cell suspension was chosen and treated according to the enclosed manual.

Varying conditions:
Step 1: Cell lysis for Gram-negative bacteria: 190 µl TE buffer + 10 µl lysozyme
(Only for *S. mutans*: Cell lysis for Gram-positive bacteria: 100 µl TE buffer + 100 µl lysozyme)
Step 6: Elution buffer volume: 75 µl

The extracted DNA was stored in Eppendorf tubes (1,5 ml; safe lock) at -30°C.

3.8 Agarose gel electrophoresis

All agarose gels were made according to the instructions given by the laboratory staff. In order to have an agarose concentration of 2%, 1,4 g of agarose were weighed in for 70 ml of gel.

The electrophoresis parameters were 80 V, 2000 mA, 20°C and the running times were 35, 40 or 50 min.

Staining was made with ethidium bromide for 20 min and destaining with TAE-buffer (0,75x) for 10 min.

The gels were then irradiated with UV light (254 nm) and photographed.

4 Results

4.1 Primer testing

The primers were tested for cross-reactions and unspecific amplification products in assays containing one of the specific primer pairs and 1 µl of a DNA mix. No unspecific products or cross-reactions were found.

The PCR amplification was followed by agarose gel electrophoresis under the previously stated conditions. All assays were made in duplicate.

Table 6: In the following figures, abbreviations were used due to space restrictions. This table shows the corresponding strains and amplicon lengths.

Strain	Amplicon length	Primer abbreviation
Aggregatibacter actinomycetemcomitans	276 bp	Aa 276
	557 bp	Aa 557
Campylobacter rectus	598 bp	Cr 598
Eikenella corrodens	58 bp	Ec 58
	688 bp	Ec 688
Porphyromonas gingivalis	404 bp	Pg 404
	437 bp	Pg 437
Prevotella intermedia	86 bp	Pi 86
	575 bp	Pi 575
Prevotella. nigrescens	804 bp	Pn 804
Streptococcus mutans	90 bp	Sm 90
	479 bp	Sm 479
Treponema denticola	63 bp	Td 63
	316 bp	Td 316
Tannerella forsythia	127 bp	Tf 127
	641 bp	Tf 641

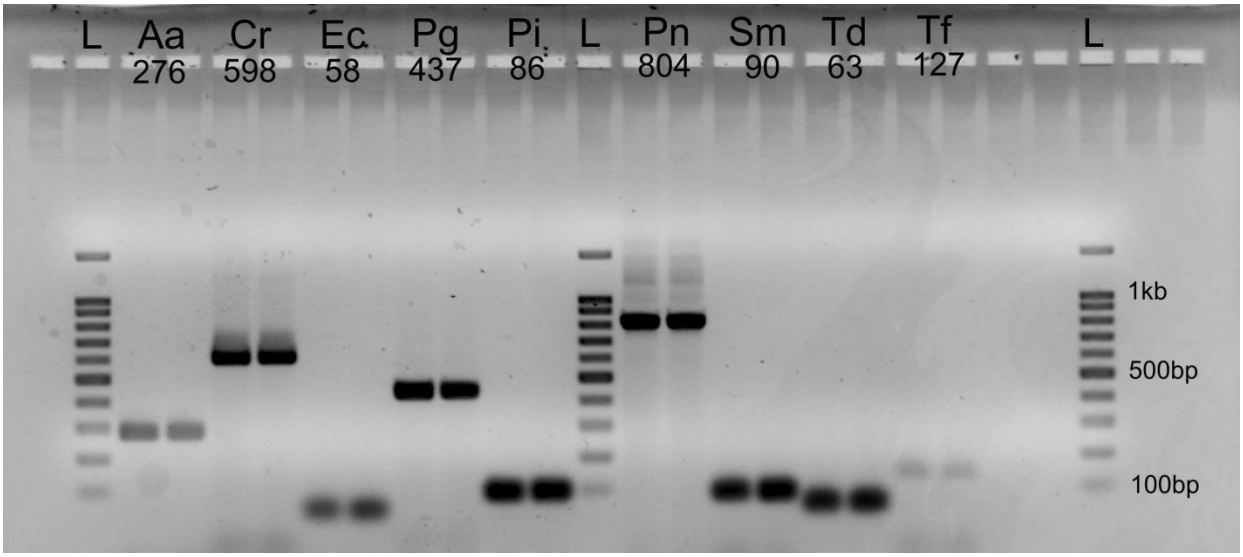

Figure 5: The single bands for each primer system confirm their specificity. All amplified PCR products had the expected size. No unspecific products were detected. In case of Tf, bands are weak – a higher cycle number would be recommended. (L=ladder)

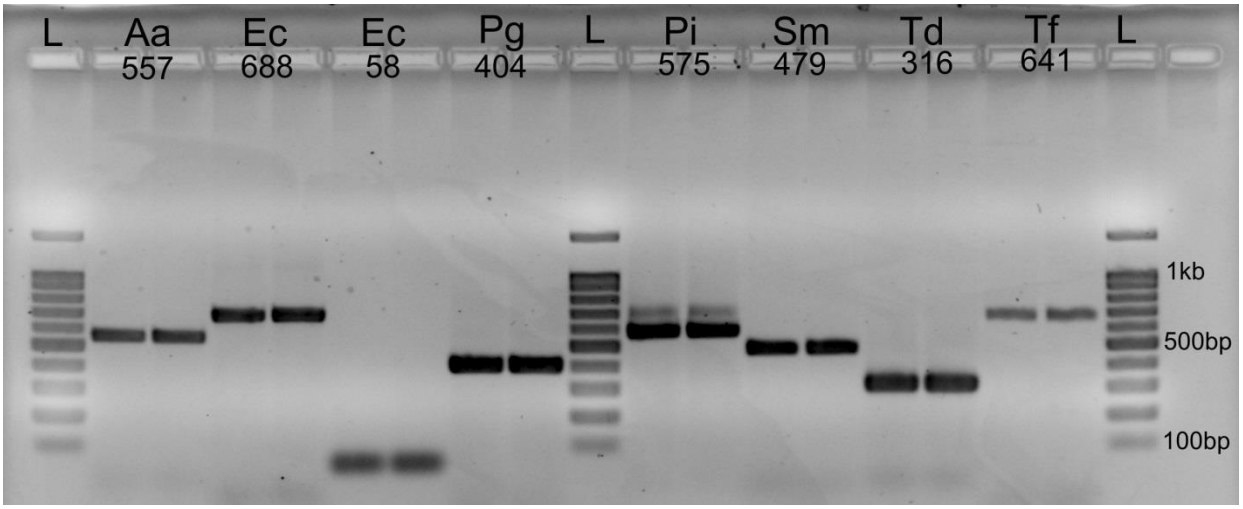

Figure 6: The single bands for each primer system confirm their specificity. All amplified PCR products had the expected size. No unspecific products were detected. (L=ladder)

4.2 Real-time PCR

4.2.1 *A. actinomycetemcomitans*

Primer pair Aa557F/Aa557R

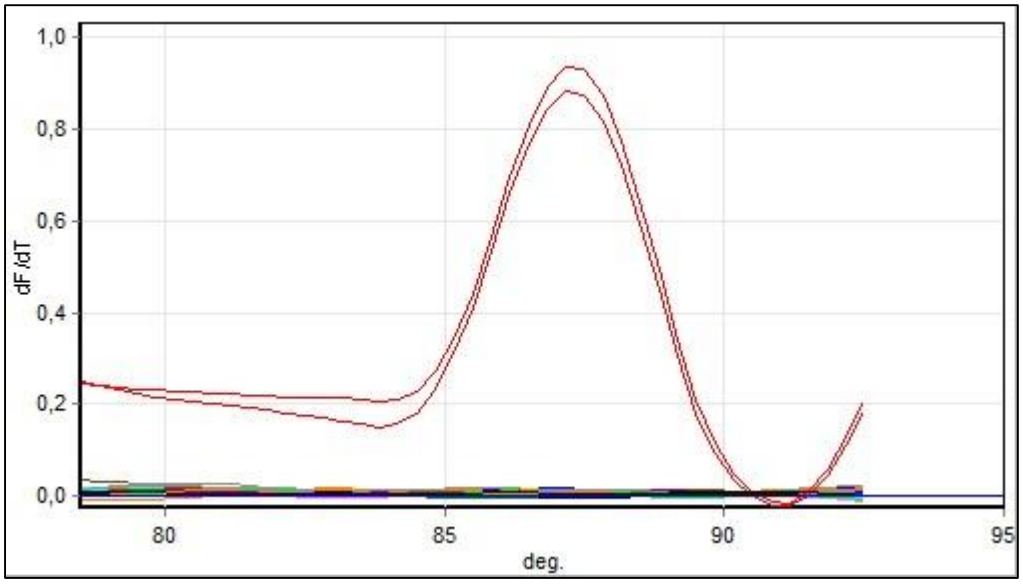

Figure 7: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *A. actinomycetemcomitans* producing a 557 bp amplicon.

Melting curve analysis shows a peak at 87,3°C for the positive control. None of the samples showed positive results.

Primer pair Aa276F/Aa276R

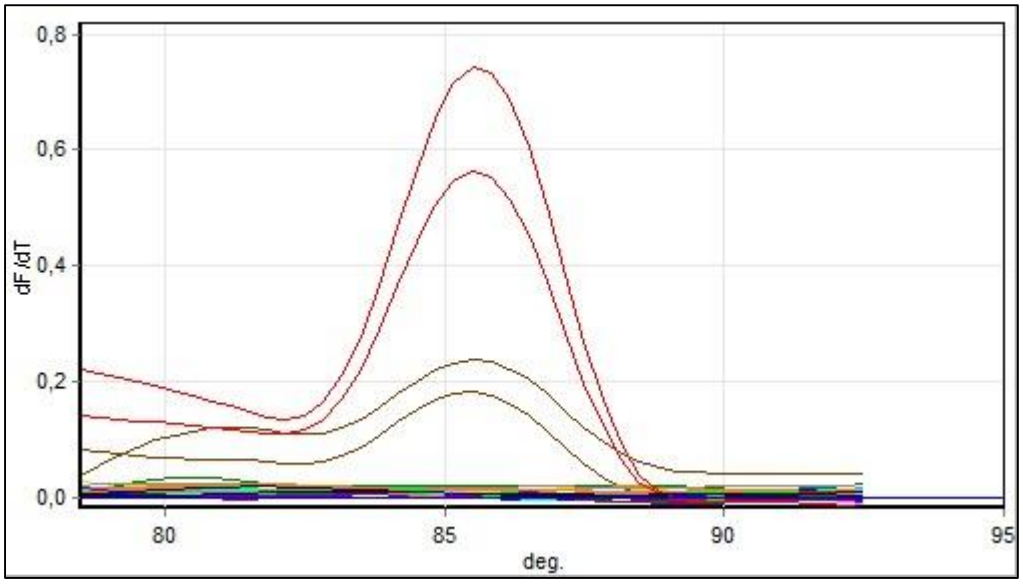

Figure 8: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *A. actinomycetemcomitans* producing a 276 bp amplicon.

Melting curve analysis shows a peak at 85,5°C for the positive control (in red). One sample showed a positive result.

Table 7: Melting curve peak values for positive samples of assay Aa 276.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
7	85,5°C	85,5°C	85,5°C	±0°C

4.2.2 *C. rectus*

Primer pair Cr598F/598R

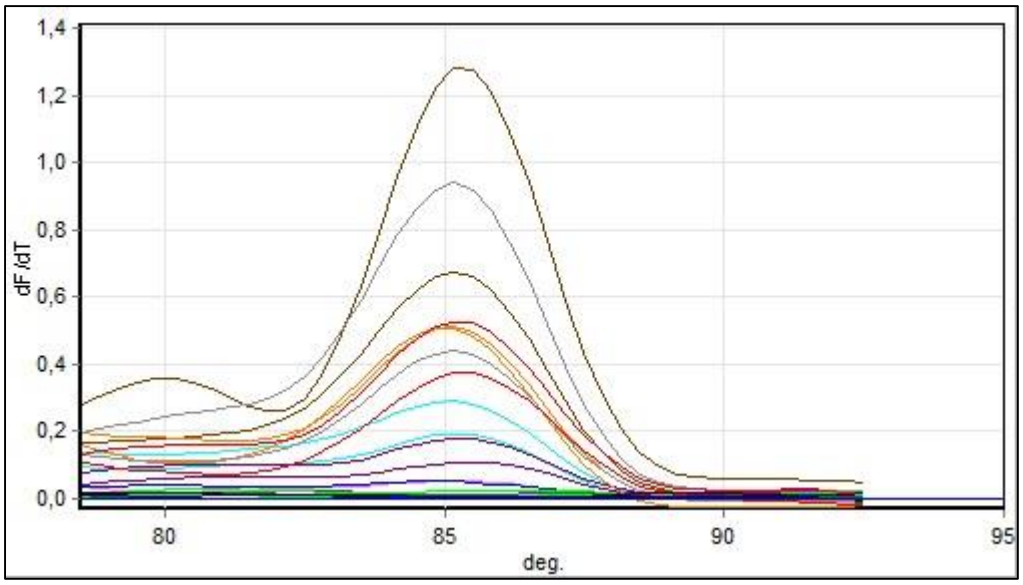

Figure 9: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *C.rectus* producing a 598 bp amplicon.

Melting curve analysis shows a peak at 85,3°C for the positive control (in red). Five samples showed a positive result.

Table 8: Melting curve peak values for positive samples of assay Cr 598.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
4	85,2°C	85,0°C	85,1°C	-0,2°C
7	85,2°C	85,3°C	85,3°C	±0°C
8	85,3°C	85,5°C	85,4°C	+0,1°C
9	85,0°C	85,0°C	85,0°C	-0,3°C
10	85,2°C	85,2°C	85,2°C	-0,1°C

4.2.3 *E. corrodens*

Primer pair Ec688F/Ec688R

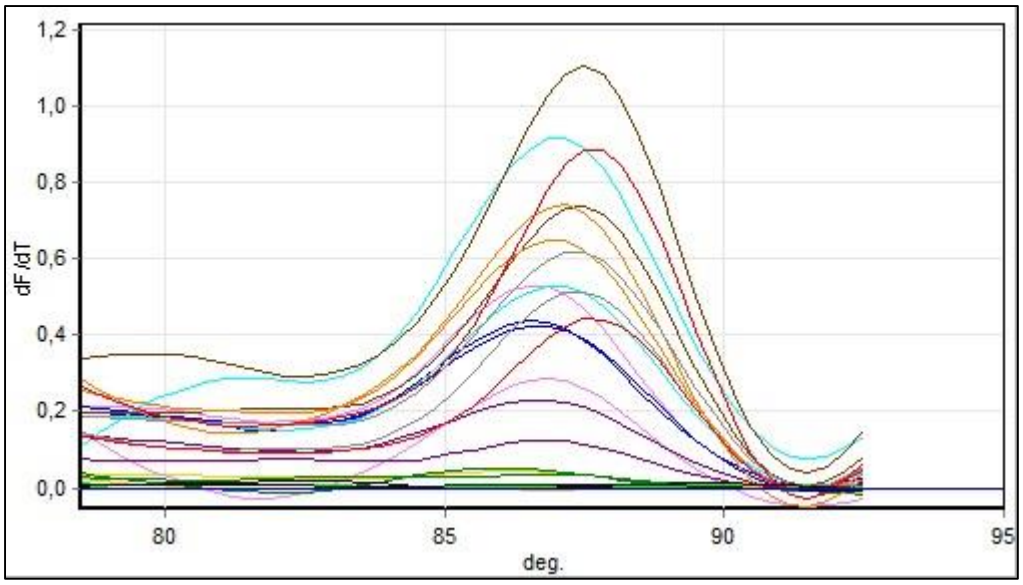

Figure 10: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *E. corrodens* producing a 688 bp amplicon.

Melting curve analysis shows a peak at 87,7°C for the positive control (in red). Seven samples showed a positive result.

Table 9: Melting curve peak values for positive samples of assay Ec 688.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
2	86,5°C	86,8°C	86,7°C	-1,0°C
3	86,7°C	86,5°C	86,6°C	-1,1°C
4	87,0°C	87,0°C	87,0°C	-0,7°C
7	87,5°C	87,5°C	87,5°C	-0,2°C
8	86,8°C	86,7°C	86,8°C	-0,9°C
9	87,0°C	87,0°C	87,0°C	-0,7°C
10	87,3°C	87,3°C	87,3°C	-0,4°C

Primer pair Ec58F/Ec58R

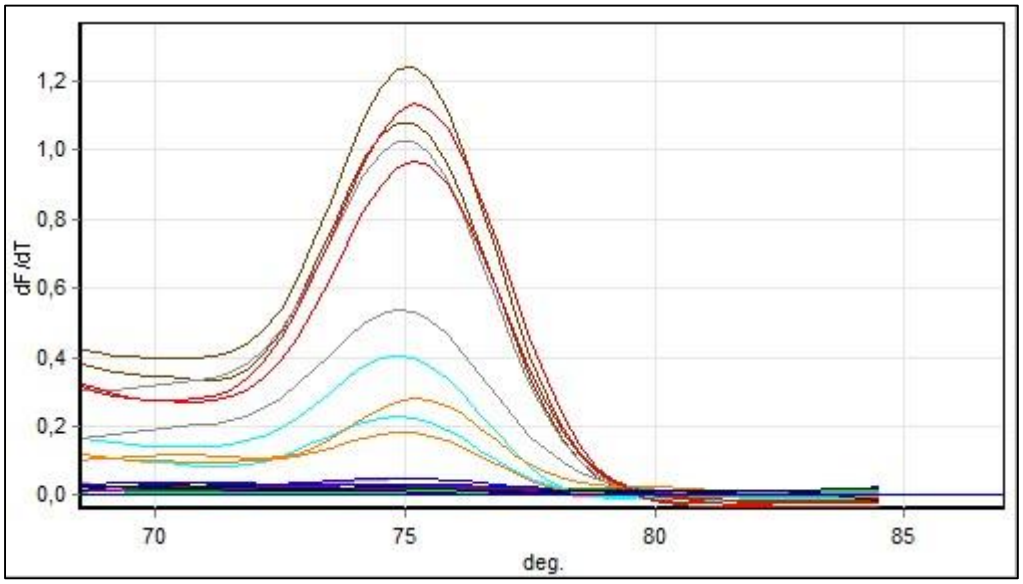

Figure 11: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *E. corrodens* producing a 58 bp amplicon.

Melting curve analysis shows a peak at 75,2°C for the positive control (in red). Four samples showed a positive result.

Table 10: Melting curve peak values for positive samples of assay Ec 58.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
4	74,8°C	74,8°C	74,8°C	-0,4°C
7	75,0°C	75,0°C	75,0°C	-0,2°C
9	75,3°C	75,0°C	75,2°C	±0°C
10	75,0°C	75,0°C	75,0°C	-0,2°C

4.2.4 *P. gingivalis*

Primer pair Pg1132/Pg729

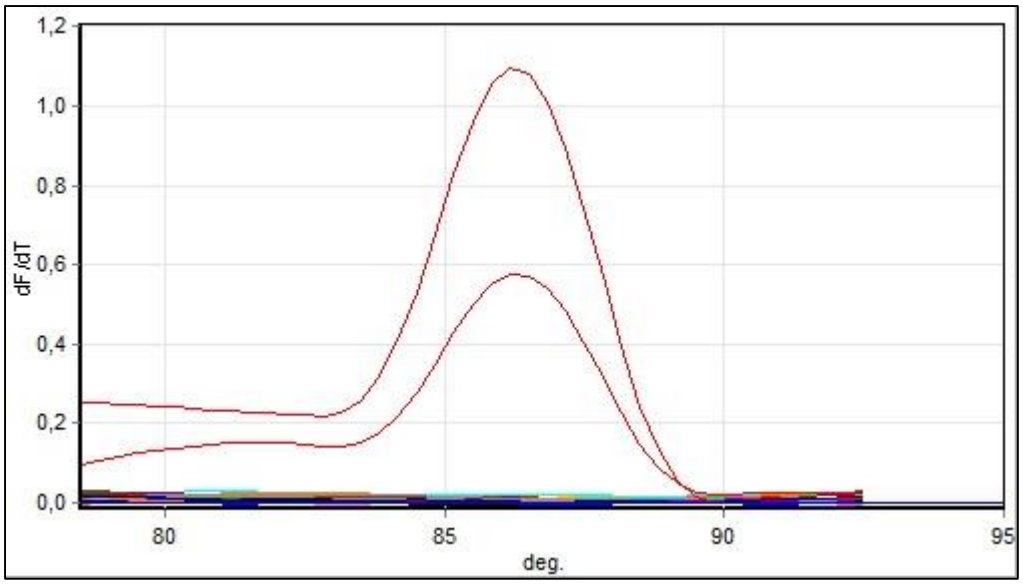

Figure 12: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *P. gingivalis* producing a 404 bp amplicon.

Melting curve analysis shows a peak at 86,3°C for the positive control. None of the samples showed positive results.

Primer pair Pg437F/Pg437R

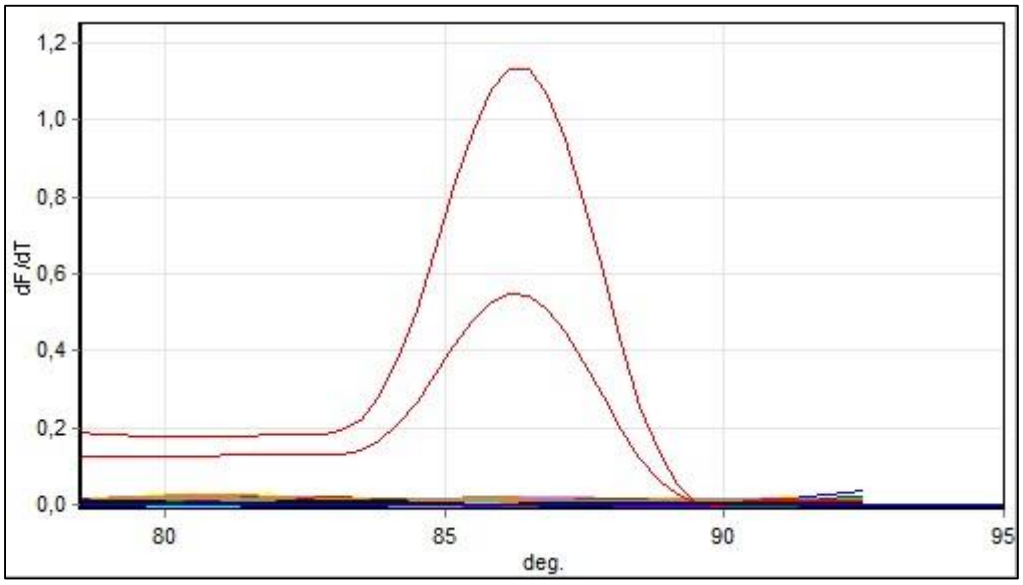

Figure 13: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *P. gingivalis* producing a 437 bp amplicon.

Melting curve analysis shows a peak at 86,3°C for the positive control. None of the samples showed positive results.

4.2.5 *P. intermedia*

Primer pair Pi575F/Pi575R

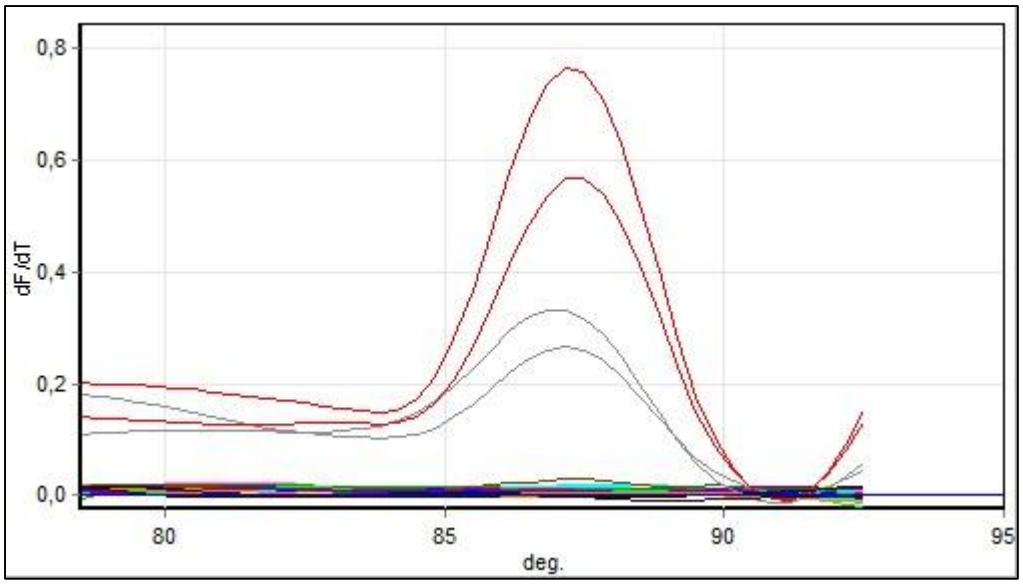

Figure 14: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *P. intermedia* producing a 575 bp amplicon.

Melting curve analysis shows a peak at 87,3°C for the positive control (in red). One sample showed a positive result.

Table 11: Melting curve peak values for positive samples of assay Pi 575.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
10	87,2°C	87,0°C	87,1°C	-0,2°C

Primer pair Pi86F/Pi86R

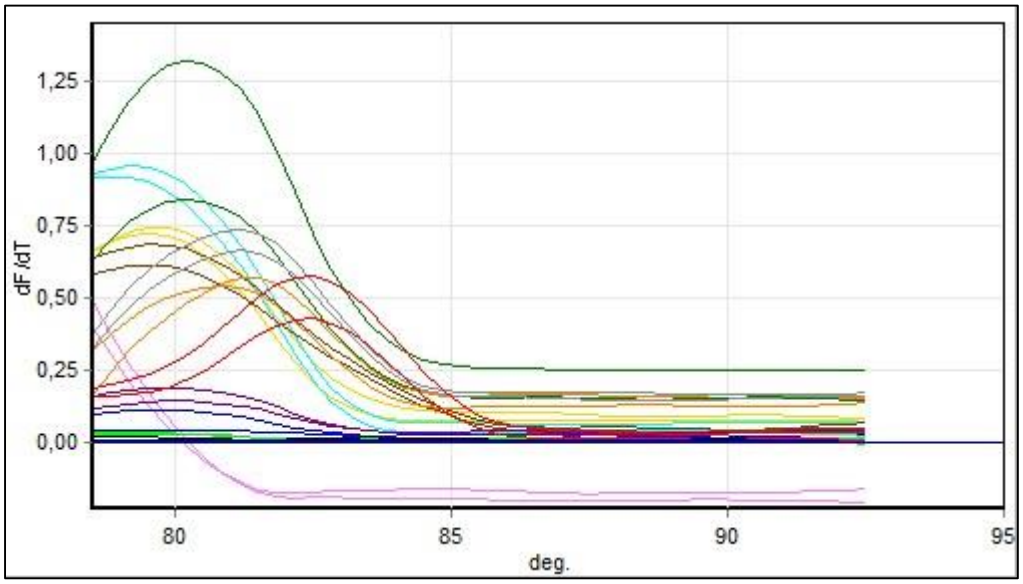

Figure 15: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *P. intermedia* producing a 86 bp amplicon.

Melting curve analysis shows a peak at 82,5°C for the positive control (in red). Six samples showed positive results, even though large temperature differences between the peaks are to be noted in some cases.

Table 12: Melting curve peak values for positive samples of assay Pi 86.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
1	79,5°C	79,7°C	79,6°C	-2,9°C
4	79,3°C	79,0°C	79,2°C	-3,3°C
5	80,2°C	80,2°C	80,2°C	-2,3°C
7	79,5°C	79,5°C	79,5°C	-3,0°C
9	80,8°C	81,3°C	81,1°C	-1,4°C
10	81,0°C	81,2°C	81,1°C	-1,4°C

4.2.6 *P. nigrescens*

Primer pair Pn804F/Pn804R

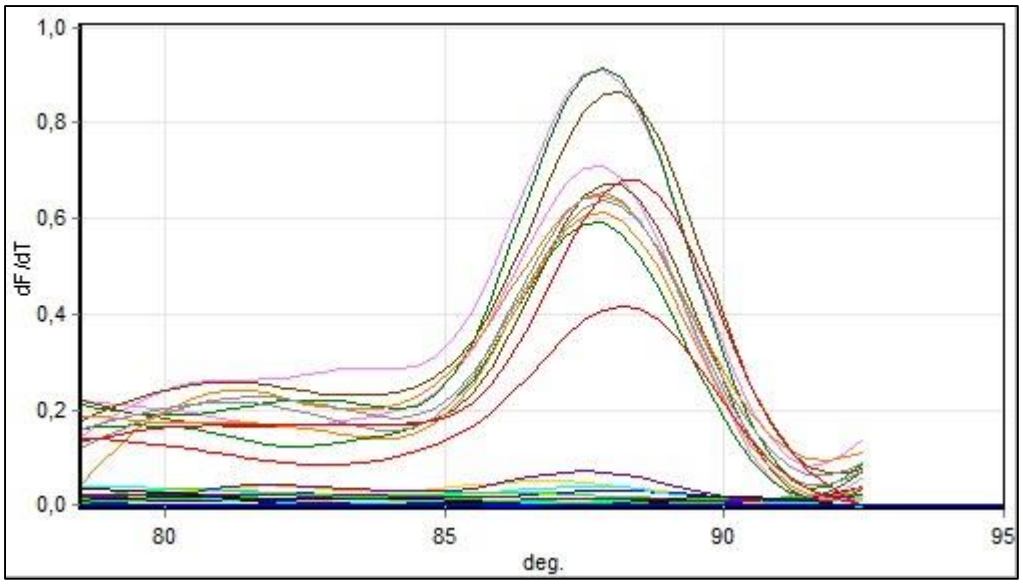

Figure 16: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *P. nigrescens* producing a 804 bp amplicon.

Melting curve analysis shows a peak at 88,3°C for the positive control (in red). Five samples showed a positive result.

Table 13: Melting curve peak values for positive samples of assay Pn 804.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
2	87,7°C	87,8°C	87,8°C	-0,5°C
5	87,8°C	87,7°C	87,8°C	-0,5°C
7	88,0°C	88,0°C	88,0°C	-0,3°C
9	87,8°C	87,8°C	87,8°C	-0,5°C
10	87,8°C	87,8°C	87,8°C	-0,5°C

4.2.7 *S. mutans*

Primer pair Sm90F/Sm90R

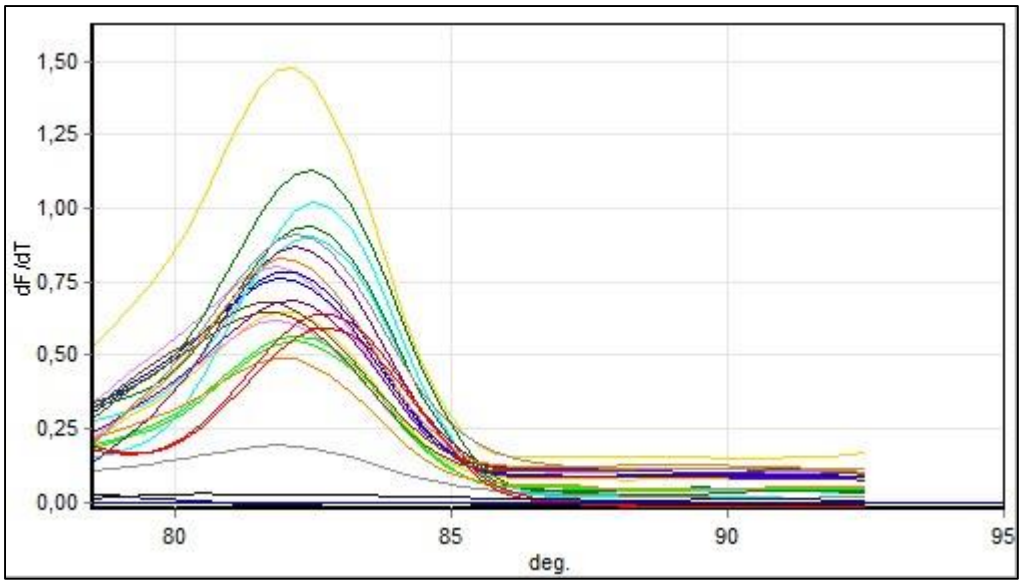

Figure 17: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *S. mutans* producing a 90 bp amplicon.

Melting curve analysis shows a peak at 82,7°C for the positive control (in red). All samples showed a positive result.

Table 14: Melting curve peak values for positive samples of assay Sm 90.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
1	82,0°C	82,0°C	82,0°C	-0,7°C
2	81,8°C	81,8°C	81,8°C	-1,1°C
3	82,0°C	82,0°C	82,0°C	-0,7°C
4	82,5°C	82,5°C	82,5°C	-0,2°C
5	82,5°C	82,5°C	82,5°C	-0,2°C
6	82,2°C	82,2°C	82,2°C	-0,5°C
7	81,7°C	81,7°C	81,7°C	-1,0°C
8	82,0°C	82,2°C	82,1°C	-0,6°C
9	82,0°C	82,0°C	82,0°C	-0,7°C
10	81,8°C	82,2°C	82,0°C	-0,7°C

Primer pair Sm479F/Sm479R

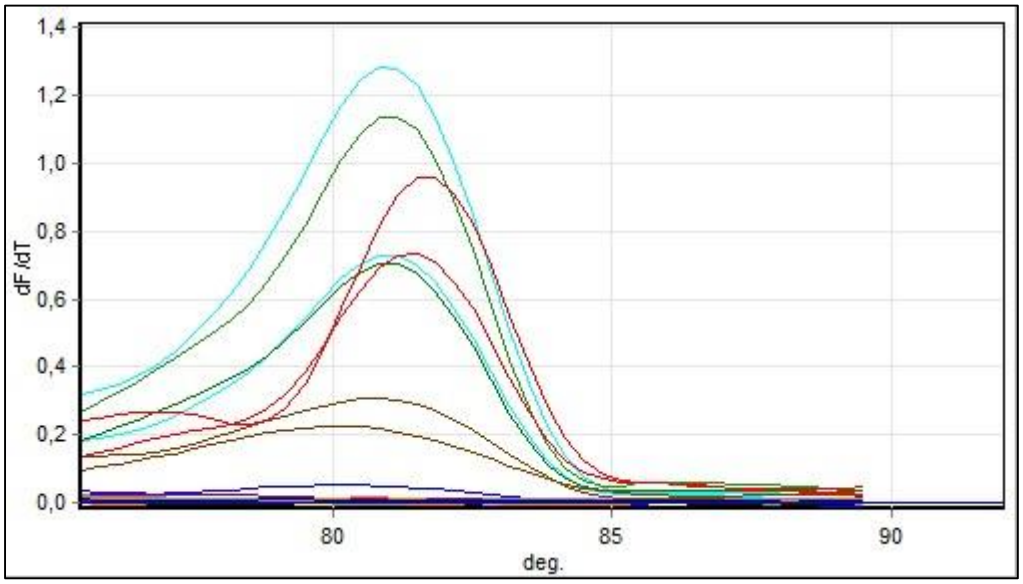

Figure 18: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *S. mutans* producing a 479 bp amplicon.

Melting curve analysis shows a peak at 81,6°C for the positive control (in red). Three samples showed a positive result.

Table 15: Melting curve peak values for positive samples of assay Sm 479.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
4	81,0°C	81,0°C	81,0°C	-0,6°C
5	81,0°C	81,0°C	81,0°C	-0,6°C
7	80,2°C	80,7°C	80,5°C	-1,1°C

4.2.8 *T. denticola*

Primer pair Td316F/Td316R

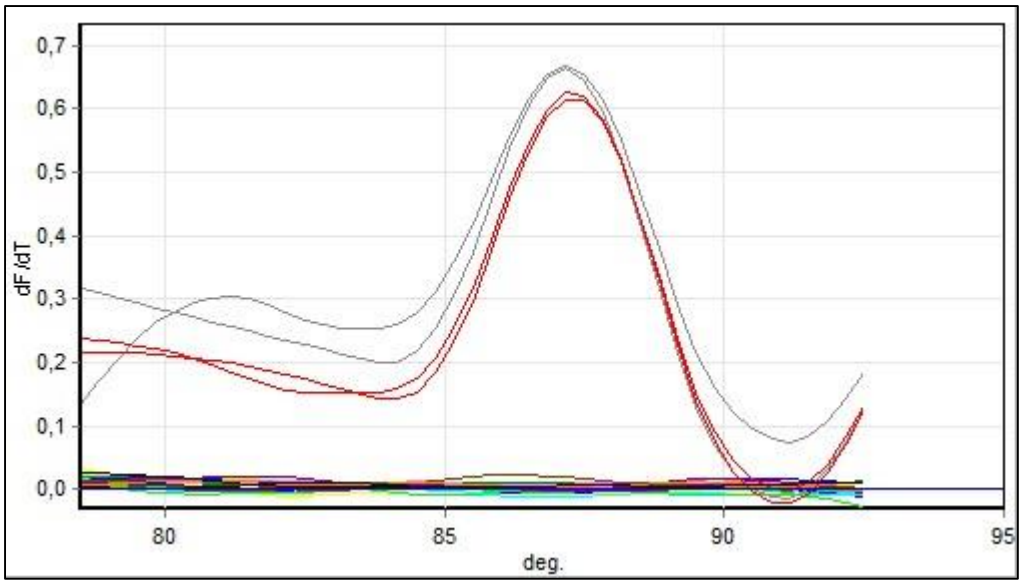

Figure 19: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *T. denticola* producing a 316 bp amplicon.

Melting curve analysis shows a peak at 87,3°C for the positive control (in red). One sample showed a positive result.

Table 16: Melting curve peak values for positive samples of assay Td 316.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
10	87,2°C	87,2°C	87,2°C	-0,1°C

Primer pair Td63F/Td63R

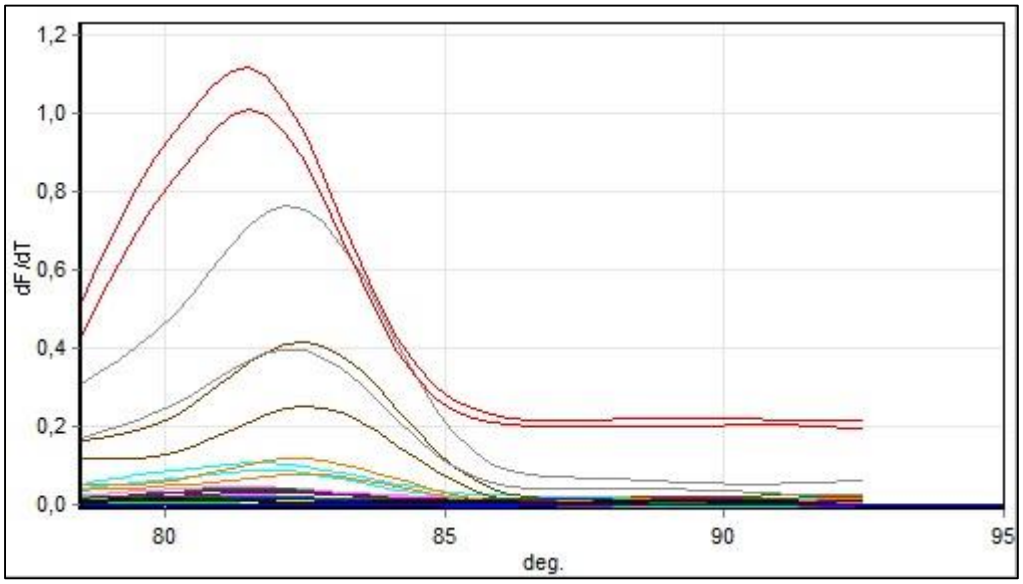

Figure 20: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *T. denticola* producing a 63 bp amplicon.

Melting curve analysis shows a peak at 81,5°C for the positive control (in red). Two samples showed a positive result.

Table 17: Melting curve peak values for positive samples of assay Td 63.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
7	82,5°C	82,5°C	82,5°C	+1,0°C
10	82,2°C	82,2°C	82,2°C	+0,7°C

4.2.9 *T. forsythia*

Primer pair Tf127F/Tf127R

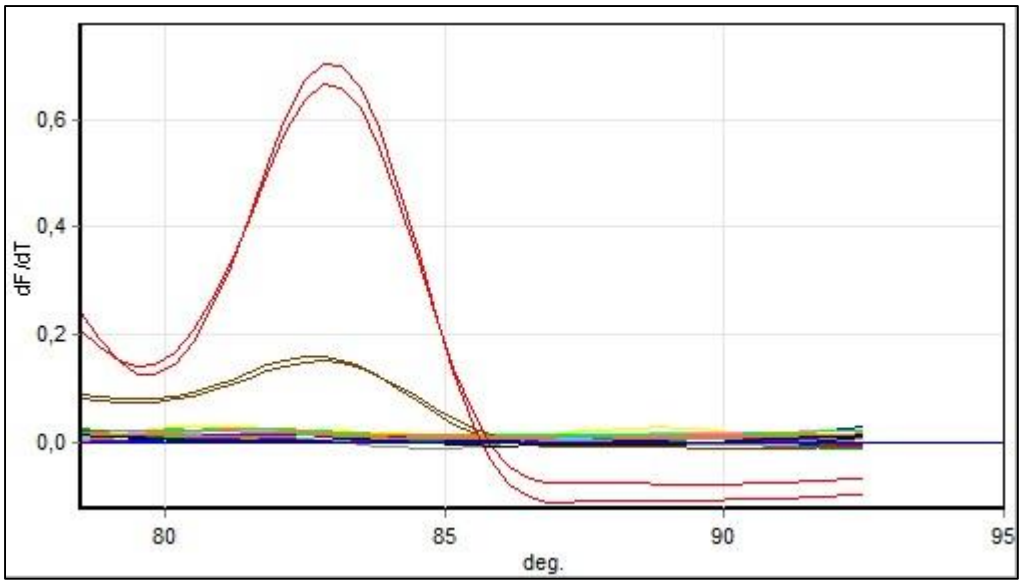

Figure 21: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *T. forsythia* producing a 127 bp amplicon.

Melting curve analysis shows a peak at 83,0°C for the positive control (in red). One sample showed a positive result.

Table 18 Melting curve peak values for positive samples of assay Tf 127.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
7	82,8°C	82,7°C	82,8°C	-0,2°C

Primer pair Bf641F/Bf641R

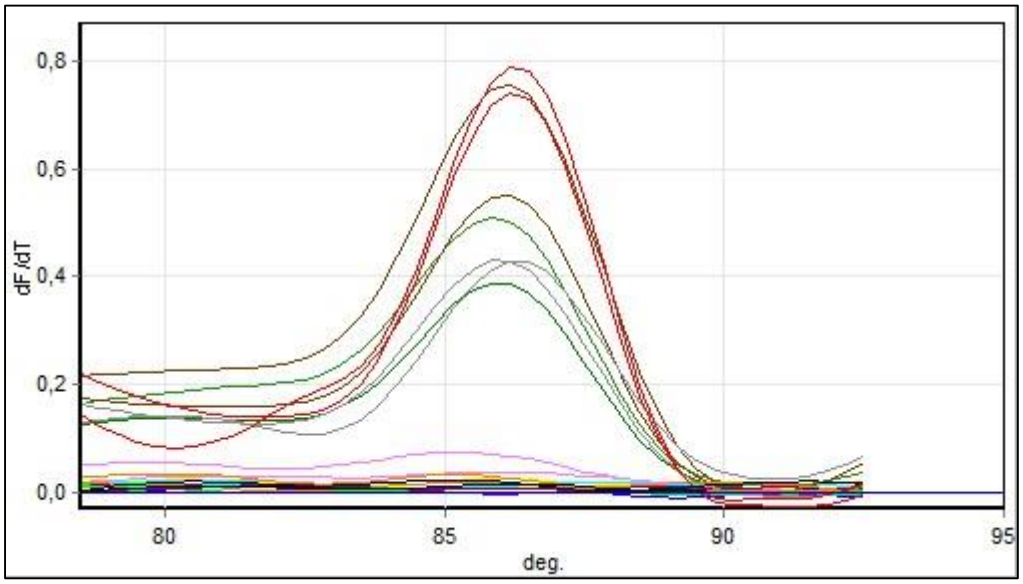

Figure 22: Melting curve profile of 10 samples and a positive control after real-time PCR amplification with a specific primer pair for *A. actinomycetemcomitans* producing a 641 bp amplicon.

Melting curve analysis shows a peak at 86,3°C for the positive control (in red). Three samples showed a positive result.

Table 19: Melting curve peak values for positive samples of assay Tf 641.

Sample nr.	Peak 1	Peak 2	Mean value	Difference
5	85,8°C	86,0°C	85,9°C	-0,4°C
7	86,0°C	86,0°C	86,0°C	-0,3°C
10	86,3°C	86,0°C	86,2°C	-0,1°C

4.3 Agarose gel electrophoresis

4.3.1 *A. actinomycetemcomitans*

Primer pair Aa557F/Aa557R

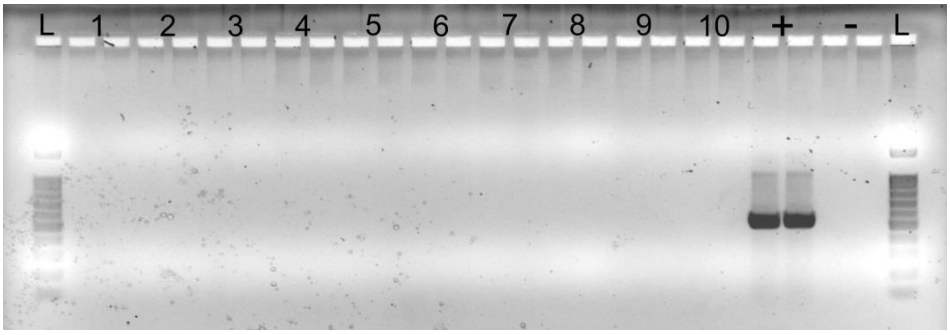

Figure 23: Agarose gel electrophoresis (40 min) of the amplification products after real-time PCR with a specific primer pair for *A. actinomycetemcomitans* producing a 557 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 557 bp for the positive control (+). None of the samples show positive results.

Primer pair Aa276F/Aa276R

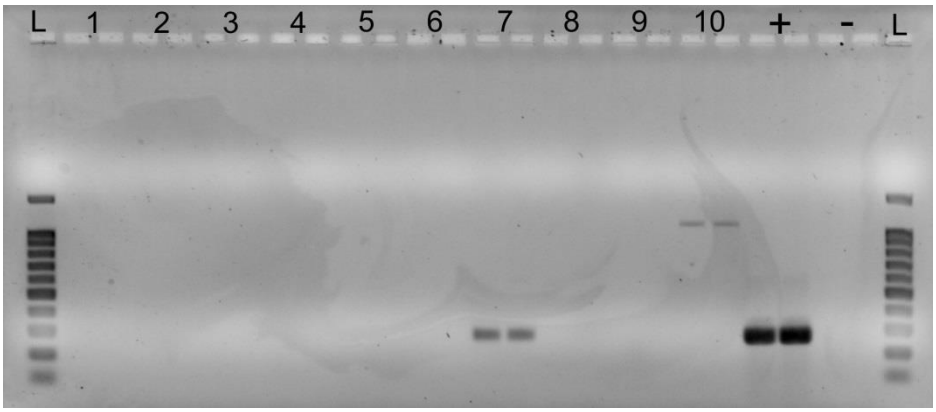

Figure 24: Agarose gel electrophoresis (50 min) of the amplification products after real-time PCR with a specific primer pair for *A. actinomycetemcomitans* producing a 276 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 276 bp for the positive control (+). Sample 7 also shows bands of the expected size. Sample 10 shows light unspecific bands of approximately 1 kb.

4.3.2 *C. rectus*

Primer pair Cr598F/Cr598R

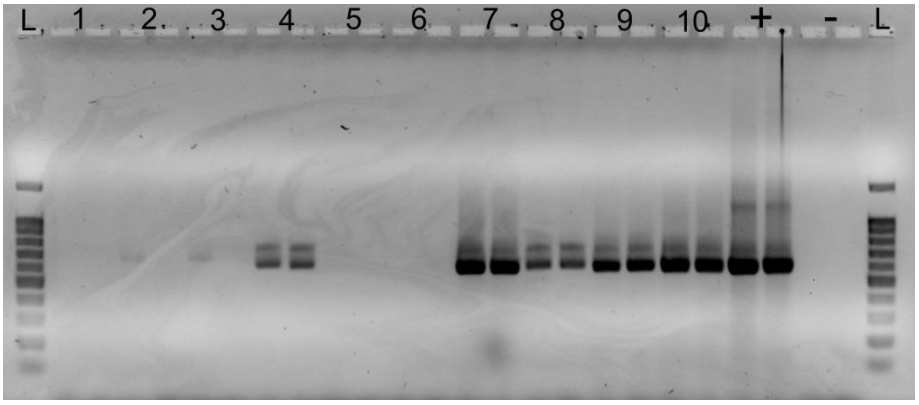

Figure 25: Agarose gel electrophoresis (50 min) of the amplification products after real-time PCR with a specific primer pair for *C. rectus* producing a 598 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 598 bp for the positive control (+). Samples 4, 7, 8, 9 and 10 also show specific bands of the expected size.

4.3.3 *E. corrodens*

Primer pair Ec688F/Ec688R

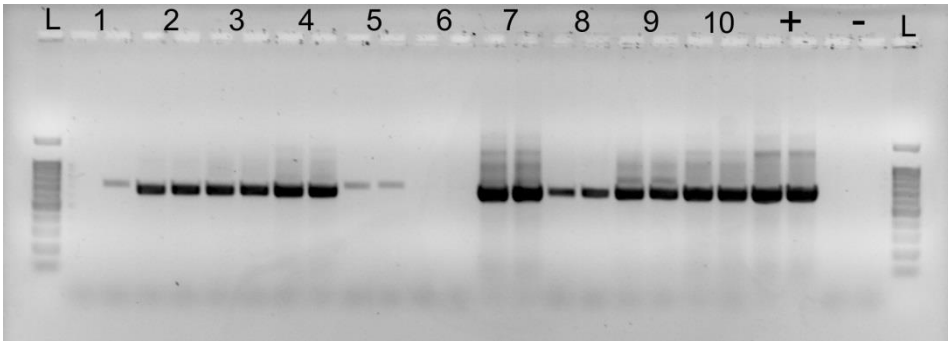

Figure 26: Agarose gel electrophoresis (35 min) of the amplification products after real-time PCR with a specific primer pair for *E. corrodens* producing a 688 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 688 bp for the positive control (+). Samples 2, 3, 4, 7, 8, 9 and 10 also show specific bands of the expected size. Sample 5 shows light unspecific bands of approximately 600 bp. Sample 1 shows a similar band in only one of the two lanes.

Primer pair Ec58F/Ec58R

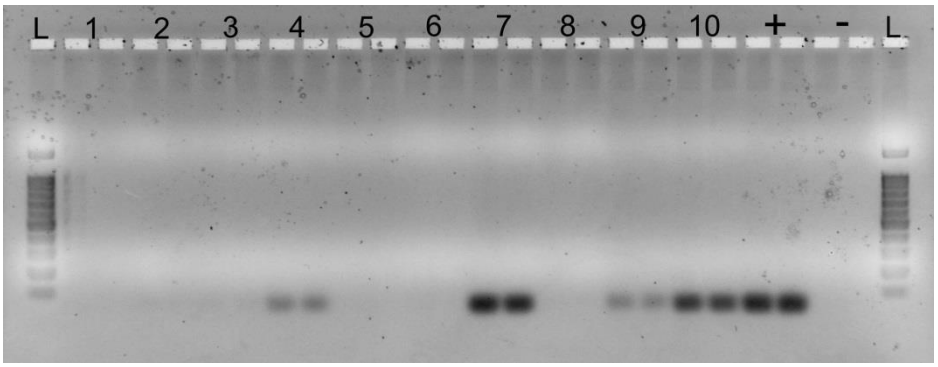

Figure 27: Agarose gel electrophoresis (40 min) of the amplification products after real-time PCR with a specific primer pair for *E. corrodens* producing a 58 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 58 bp for the positive control (+). Samples 4, 7, 9 and 10 also show specific bands of the expected size.

4.3.4 *P. gingivalis*

Primer pair Pg1132/Pg729

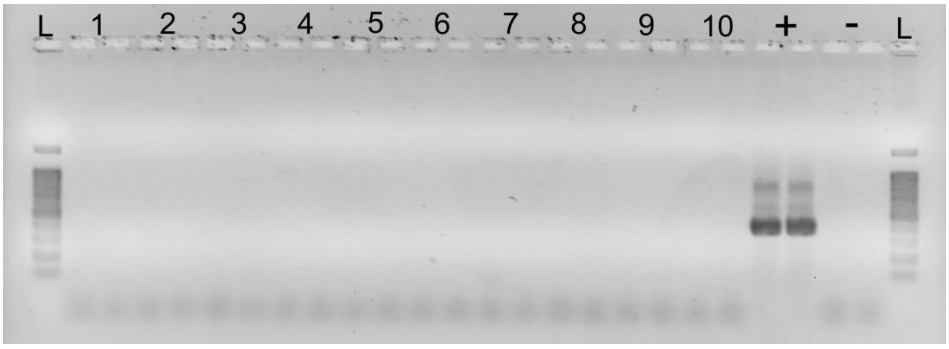

Figure 28: Agarose gel electrophoresis (35 min) of the amplification products after real-time PCR with a specific primer pair for *P. gingivalis* producing a 404 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 404 bp for the positive control (+). None of the samples show positive results.

Primer pair Pg437F/Pg437R

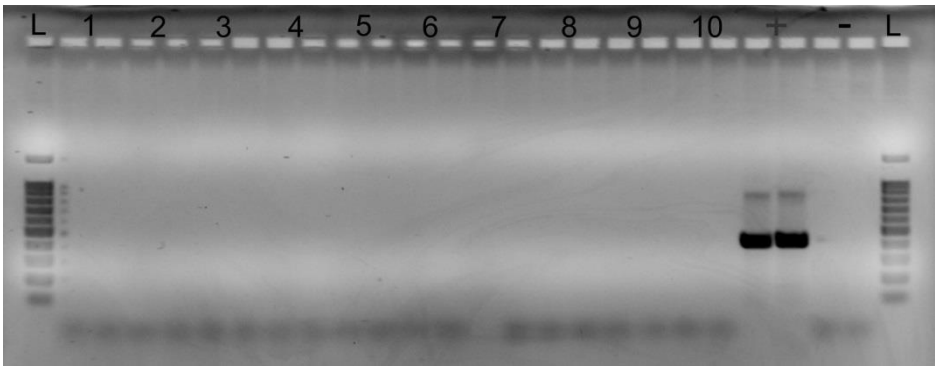

Figure 29: Agarose gel electrophoresis (40 min) of the amplification products after real-time PCR with a specific primer pair for *P. gingivalis* producing a 437 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 437 bp for the positive control (+). None of the samples show positive results.

4.3.5 *P. intermedia*

Primer pair Pi575F/Pi575R

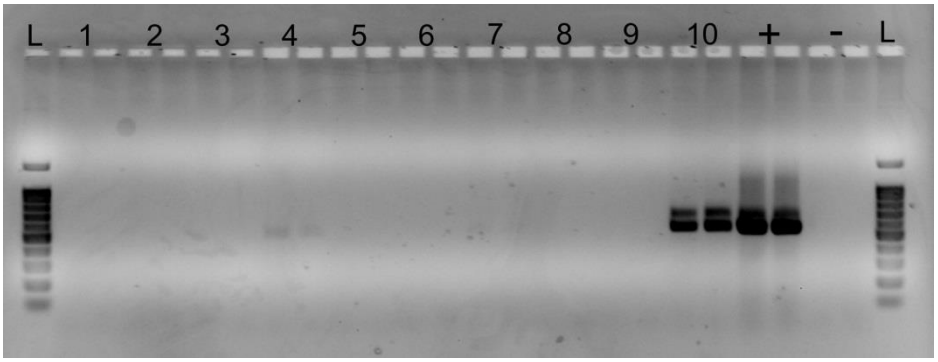

Figure 30: Agarose gel electrophoresis (40 min) of the amplification products after real-time PCR with a specific primer pair for *P. inte,media* producing a 575 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 575 bp for the positive control (+). Sample 10 also shows specific bands of the expected size.

Primer pair Pi86F/Pi86R

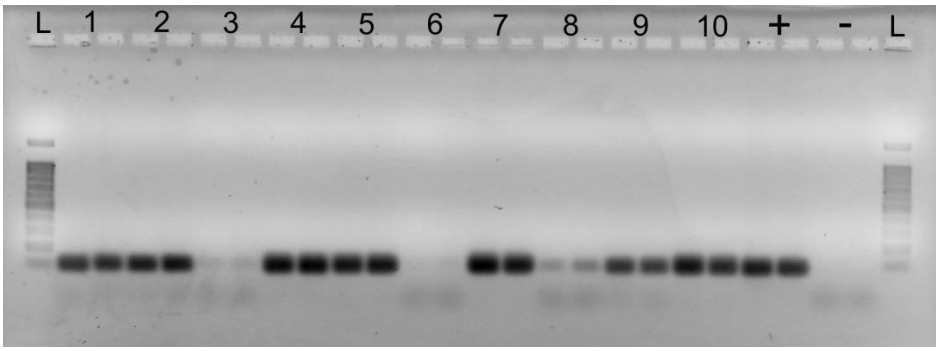

Figure 31: Agarose gel electrophoresis (35 min) of the amplification products after real-time PCR with a specific primer pair for *P. intermedia* producing a 86 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 86 bp for the positive control (+). Almost all samples (1, 2, 4, 5, 7, 8, 9 and 10) also show specific bands of the expected size.

4.3.6 *P. nigrescens*

Primer pair Pn804F/Pn804R

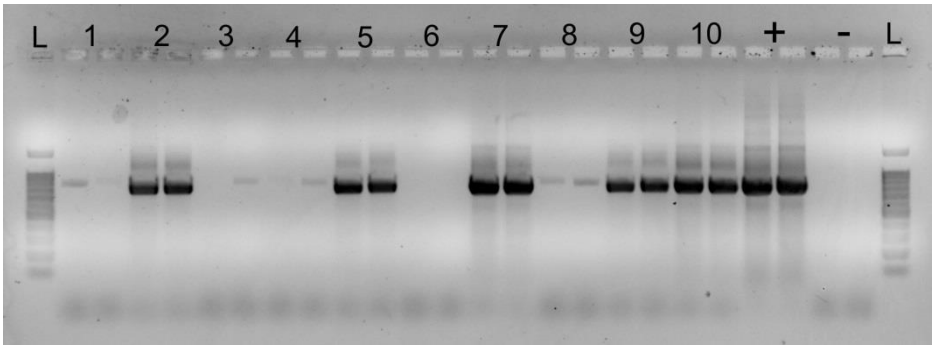

Figure 32: Agarose gel electrophoresis (35 min) of the amplification products after real-time PCR with a specific primer pair for *P. nigrescens* producing a 804 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 804 bp for the positive control (+). Samples 2, 5, 7, 9 and 10 also show specific bands of the expected size. Lighter, unspecific bands are detected in one lane of samples 1, 3 and 4 and in both lanes of sample 8.

4.3.7 *S. mutans*

Primer pair Sm479F/Sm479R

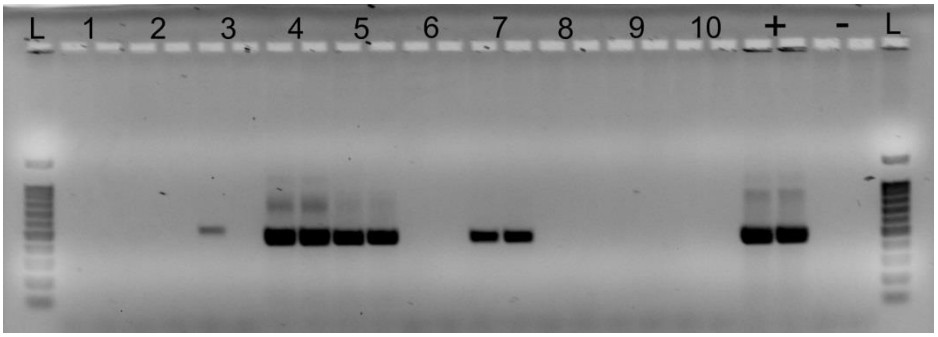

Figure 33: Agarose gel electrophoresis (40 min) of the amplification products after real-time PCR with a specific primer pair for *S. mutans* producing a 479 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 479 bp for the positive control (+). Samples 4, 5 and 7 also show specific bands of the expected size. One small unspecific band is detected in one lane of sample 3.

Primer Pair Sm90F/Sm90R

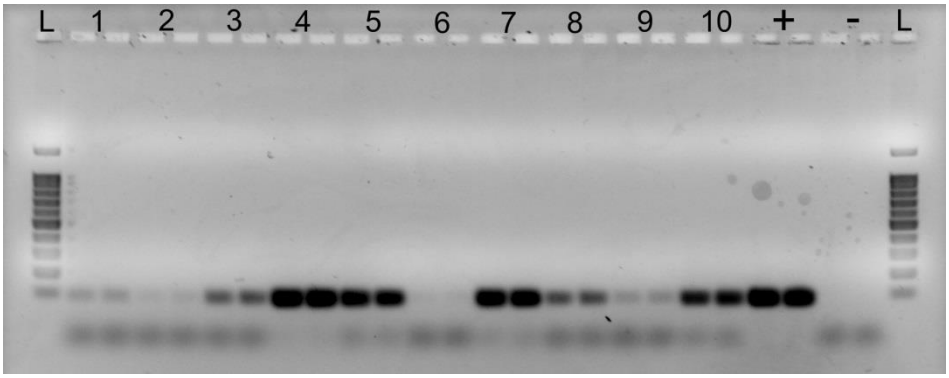

Figure 34: Agarose gel electrophoresis (40 min) of the amplification products after real-time PCR with a specific primer pair for *S. mutans* producing a 90 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 90 bp for the positive control (+). Almost all samples (1, 3, 4, 5, 7, 8, 9 and 10) also show specific bands of the expected size.

4.3.8 *T. denticola*

Primer pair Td316F/Td316R

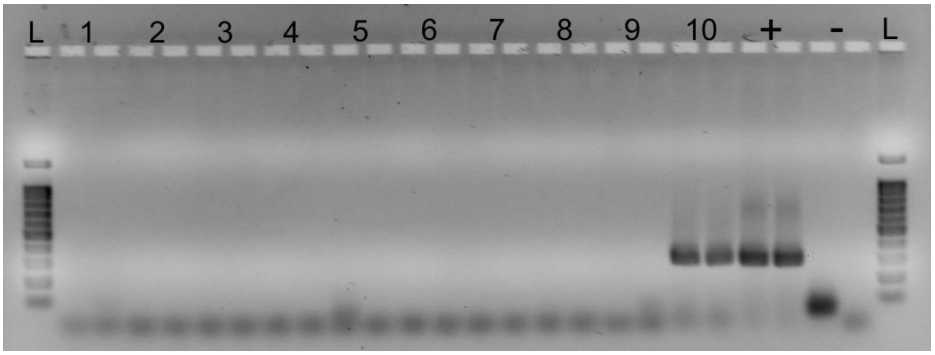

Figure 35: Agarose gel electrophoresis (40 min) of the amplification products after real-time PCR with a specific primer pair for *T. denticola* producing a 316 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 316 bp for the positive control (+). Sample 10 also shows specific bands of the expected size. A dark stain is to be noted at the bottom of one lane of the negative control.

Primer pair Td63F/Td63R

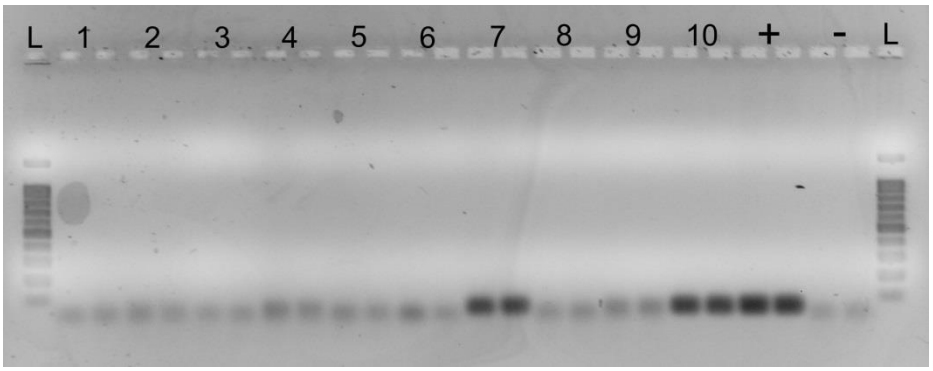

Figure 36: Agarose gel electrophoresis (40 min) of the amplification products after real-time PCR with a specific primer pair for *T. denticola* producing a 63 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 63 bp for the positive control (+). Samples 7 and 10 also show specific bands of the expected size.

4.3.9 *T. forsythia*

Primer pair Bf641F/Bf641R

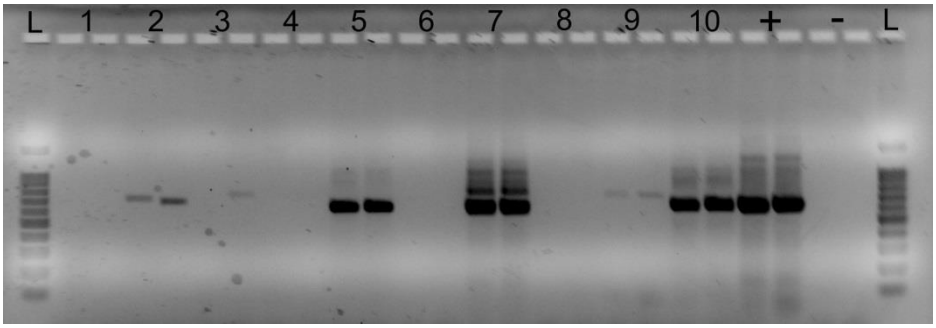

Figure 37: Agarose gel electrophoresis (40 min) of the amplification products after real-time PCR with a specific primer pair for *T. forsythia* producing a 641 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 641 bp for the positive control (+). Samples 5, 7 and 10 also show specific bands of the expected size. Lighter, unspecific bands are to be noted in both lanes of samples 2 and 9, and in one lane of sample 3.

Primer pair Tf127F/Tf127R

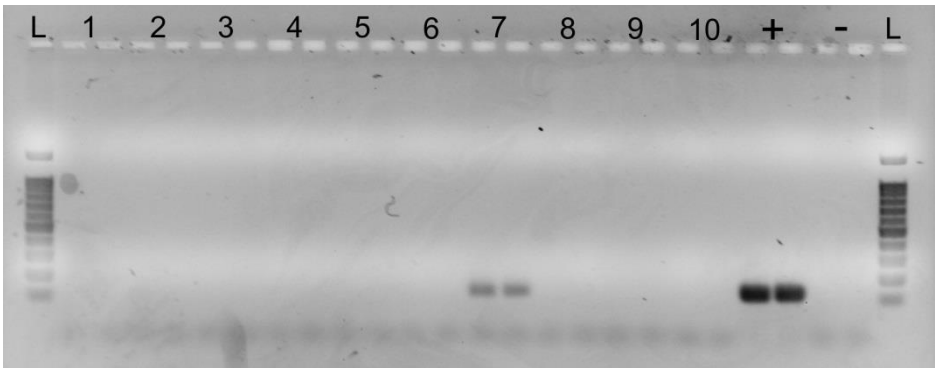

Figure 38: Agarose gel electrophoresis (40 min) of the amplification products after real-time PCR with a specific primer pair for *T. forsythia* producing a 127 bp amplicon. (-=negative control, L=ladder)

Electrophoresis shows specific bands of the expected size of 127 bp for the positive control (+). Sample 7 also shows specific bands of the expected size.

4.4 Summary

sample nr.	1	2	3	4	5	6	7	8	9	10
Aa 557										
Aa 276							x			
Cr 589				x			x	x	x	x
Ec 58				x			x		x	x
Ec 688		x	x	x			x	x	x	x
Pg 404										
Pg 437										
Pi 575										x
Pi 86	x			x	x		x		x	x
Pn 804		x			x		x		x	x
Sm 90	x	x	x	x	x	x	x	x	x	x
Sm 479				x	x		x			
Td 316										x
Td 63							x			x
Tf 641					x		x			x
Tf 127							x			

The results obtained through real-time PCR (marked with an x) were confirmed by the gel electrophoresis (marked in grey) in almost all cases. Inconsistencies happened during the assay Pi 86 (samples 2 and 8 showed specific bands on the agarose gel, even though the melting curve analysis would not have indicated that) and Sm 90 (samples 2 and 6 showed typical peaks in the melting curve but only very weak, almost invisible bands).

It is interesting to note that the different primer systems for the same microorganisms lead to different results. This inconsistency should be subject of further investigation, in order to find the right primer systems for accurate detection of the microorganisms in question.

4.5 Questionnaires

All questionnaires were filled in correctly. None of the participants complained about incomprehensible or confusing questions. There were also no complaints about the number of questions or their nature. The type of information asked, the complexity and duration of the

questionnaire seem to have met the participants' expectations. It also seemed like none of the participants was overstrained by the given task.

5 Discussion

Selection of microorganisms and primers

The aim of this work was to determine whether real-time PCR was a suitable method to detect certain periodontitis-related microorganisms that can be found in the oral cavity. Therefore it was necessary to find a specific primer pair for each one of them. The used primer pairs were found in different publications from different authors. At first, the focus was on *Porphyromonas gingivalis*, a well-studied pathogen related to periodontitis, but not often found in healthy subjects. (Slots, Ashimoto et al. 1995) (Mättö, Saarela et al. 1998)

It was then decided to extend the search field to other periodontitis-related pathogens (Ashimoto, Chen et al. 1996) and also include the caries-related *Streptococcus mutans*, since the participants were no longer chosen among periodontitis patients. (Mitrakul, Asvanund et al. 2011) A specific primer pair for the detection of *S. mutans* in plaque samples stated in a publication in the "Clinical Oral Investigations" journal was chosen. (Psoter, Ge et al. 2011)

Questionnaires

The questionnaires were designed in order to find out if microorganisms colonizing the oral cavity could be related to eating habits. Participants had to fill in a 24-hours-recall form, stating what they had eaten and drunk during the last day. They could also mention if this recall was representative for their usual diet, or not. In order to get an idea about the usual eating behavior of the participants, a food frequency questionnaire was added. It allows categorizing different foodstuffs in consumption frequency groups.

The questionnaires also had a section with questions concerning statistical data (gender, age, weight, etc.) and social background. It was interesting to know how often the participants eat out or if they prepare their meals themselves. Also if they were smokers or not, or had any health problems concerning the oral cavity.

Rheumatoid arthritis affects significantly more women than men and seems to be influenced by epigenetic factors. These include micro-nutrients or other dietary components. (Persson 2012) It is therefore crucial to analyse the diet of the study participants and try to find patterns. Statistical analysis could show relationships between gender, eating habits, prevalence of periodontitis and/or rheumatoid arthritis and the occurrence of certain microorganisms in plaque samples.

In this trial, all participants were between 24 and 41 years old and eight out of ten were females. But profound research can only be made with a higher number of study participants. Furthermore, the group should consist of equal numbers of women and men of different ages. In order to compare results of rheumatoid arthritis patients and healthy subjects, the study should be performed with two groups (patients and control).

The oral cavity is an excellent environment for biofilm formation and the high number of species present makes samples very interesting to analyse. But it has been shown that there are not only differences in microbial composition from person to person, but also from one type of intraoral location to another in the same subject (e.g. sub- and supragingival plaque) and even between similar types of locations (e.g. two different periodontal pockets). Also the nature of the surface on which the biofilm forms has an impact on its composition, and it is important how the teeth are cleaned. (Socransky and Haffajee 2005)

In order to get more meaningful data, the questionnaires should be supplemented by a form where study participants state the date and time of the sampling, the tooth (or teeth) concerned, the existence of periodontal pockets, interdental spaces or bleeding. It might also be interesting to know when the teeth had been cleaned last before the sampling procedure.

Sampling

The facts stated before also underline the fact that the sampling technique is crucial for the reproducibility of the experiments. In this case, the sampling was made by each subject. They were given a description of the procedure – but there was no way of controlling the handling. This means that it cannot be ruled out that the results might not perfectly reflect the real bacterial colonization. Biofilms are dynamic structures. The samples taken during a study are always just a representation of a certain state. They cannot reflect the reality at all times. It is therefore essential to examine large numbers of samples from similar and different locations. Given the heterogeneity, elaborate and extensive studies are the only way to attempt to eliminate the local and host-level factors that influence the composition of human oral biofilms. (Socransky and Haffajee 2005)

For more precision and reproducibility, the sampling should be done by one operator. It would even be preferable to work with professionals of periodontology for this purpose. In this trial, cooperation with oral surgeons was originally planned.

Selection of methods

A limiting factor for meaningful in vivo studies is the lack of rapid and specific techniques to analyse the large number of samples in a reasonable time period. Light microscopy, and later on electron microscopy, combined with immunocytochemical techniques are quite labor-intensive and time-consuming and therefore not suitable for the examination of high sample numbers. This is also valid for cultivation techniques. In addition to the time and material required, some species have not been cultured before or are simply uncultivable. (Socransky and Haffajee 2005)

Antibody-based methods (e.g. ELISA) do not require cultivation and are therefore faster and less expensive. Nevertheless, the application is limited by the number of species for which reagents have been developed. Polymerase Chain Reaction (PCR) is considered as a fast and simple method to analyse the oral microbiome. When the appropriate primers are available, it is highly specific and sensitive. Real-time PCR is even faster, given the fact that the subsequent gel electrophoresis is unnecessary, and it allows quantification. The use of probes (whole genomic DNA or oligonucleotides) is quite popular, but there is a risk of cross-reactions between closely related species and the sensitivity is lower than for PCR methods. (Socransky and Haffajee 2005)

When quantification is not required, checkerboard hybridization seems to be the best technique. It is fast, not expensive and it allows the detection of uncultivable species with high specificity. A large number of samples with a complex mixture of microorganisms can be examined. The only limitation is the need for the right DNA probes (similar to the antibodies for immunologic methods or the specific primers for PCR). (Socransky and Haffajee 2005)

Results

The mentioned inconsistencies between real-time PCR and electrophoresis can be explained as follows:

Assay Pi 86: The interpretation of the melting curve analysis is quite difficult to make, since the peak values vary highly and the peak of sample 2 seems to be out of the 78-93°C range.

Assay Sm 90: The very weak, almost invisible bands are due to the late start of the amplification in case of the samples 2 and 6. The fluorescence curves rise just very slightly at the end of the reaction (around cycles 27-28), whereas the positive control shows a high increase in fluorescence already after cycle 13.

The different results for the different primer systems detecting the same microorganism could most probably be due to the (non-varying) reaction parameters. The annealing/extension temperature used should be varied according to the amplicon length. The melting temperature of an oligonucleotide can be calculated, but the real optimum temperature for annealing should be determined experimentally. (Bustin, Benes et al. 2009)

The low detection rates of *A. actinomycetemcomitans* (0% and 10%) and *P. gingivalis* (0% and 0%) are not surprising. The participants in this trial were all periodontally healthy (at least to their own understanding). This was also found in a Dutch study with subjects with and without periodontal destruction. (Van Winkelhoff, Loos et al. 2002)

Further research

First of all, in this trial no quantification was made, although real-time PCR is a suitable technique for it. The quantitative relationship between the amount of target DNA present and the amount of PCR product can then be used to draw conclusions on the original bacterial load of the sample. In order to get quantitative results, a calibration curve with dilutions of the pure DNA in question is established.

The study subjects should be examined by a professional before taking part in the trial. Data about the medical condition should be collected and the sampling procedure should be standardized. Sampling should be done by the same operator or a team that agreed on a clearly defined procedure.

Standardization of the sampling procedure and enlargement of the trial group should be a way to eliminate the bias probably given by small size and homogenous character of the group of subjects in this trial. No representative conclusions can be drawn from the stated results due to the small number and unanswered open questions.

For further investigation, there should be more participants (or any) with periodontal disease, a control group and the method should be optimized by testing variable amplification conditions (such as temperatures, cycle numbers, etc.)

Studies related to the Human Microbiome Project mention “next Generation Sequencing” (nGS) as methods of choice. They are high-throughput techniques: fast analysis of a high number of samples at low costs, which is essential in this field of study. (Madupu, Szpakowski et al. 2013)

There are various commercial platforms for nGS, still some techniques are to be perfected, since the error rates are higher than for more traditional methods. Nevertheless, the high-throughput and lower costs make nGS very interesting and encourage further research in this direction. (Dave, Higgins et al. 2012)

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