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Interference with *Aggregatibacter actinomycetemcomitans*: colonization of epithelial cells under hydrodynamic conditions

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Introduction: Microbial interactions are considered to be important for bacterial colonization. Interactions that inhibit colonization of pathogens could possibly be used as a new treatment approach for periodontitis. The aim of this study was to test this hypothesis on soft surfaces *in vitro*, taking into account the hydrodynamic forces continuously present *in vivo*.
Methods: Cultured epithelial cells were precolonized with *Streptococcus sanguinis* KTH-4, *Streptococcus cristatus* CC5A, *Streptococcus salivarius* TOVE and *Streptococcus mitis* BMS before *Aggregatibacter actinomycetemcomitans* colonization. Experiments were performed in a modified Robbins-device-type flow cell. Bacterial colonization and the number of epithelial cells were evaluated by microbial culturing and quantitative polymerase chain reaction.
Results: The streptococci were able to inhibit *A. actinomycetemcomitans* colonization on soft tissue surfaces under flow conditions. Statistically significant differences were found between streptococcal pretreatments and the controls, with the most pronounced effect caused by *S. sanguinis*.
Conclusion: These data confirm the possibility of applying beneficial bacteria in periodontal treatment.

Key words: *Aggregatibacter actinomycetemcomitans*; bacterial interference; colonization; epithelial cells; streptococci

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Colonization of hard and soft tissue surfaces by periodontopathogens in the oral cavity is a first and crucial step in the development of periodontitis. This frequently encountered infection results in the destruction of the tooth-supporting tissues, the periodontium, and is also associated with a number of severe systemic disorders (19).

The oral biofilm of periodontitis patients shows an increased proportion of gram-negative pathogenic microorganisms like *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythensis* and *Porphyromonas gingivalis*. A lower prevalence of so-called beneficial bacteria is, however, also assumed to play an important role in the etiology of periodontitis. *In vivo*, aggressive periodontitis seems to be associated with, for example, a reduced colonization of *Streptococcus sanguinis* (23). Therefore, one could hypothesize that the application of such beneficial bacteria in the oral cavity might provide a new treatment approach to prevent or treat periodontal infections. Especially in this age of increasing antibiotic resistance of several microbial pathogens, alternative treatment approaches for bacterial infections should be explored. Recent investigations have shown that certain streptococcal species can interfere with the colonization of *A. actinomycetemcomitans* on epithelial surfaces under static conditions (25) and also with the colonization of *A. actinomycetemcomitans* on hard surfaces under hydrodynamic conditions (20). Given that the efficacy of the inhibitory strategies and the colonization process might be influenced by the hydrodynamic conditions (12), which are continuously present *in vivo*, the interaction between beneficial bacteria and pathogens *in vitro* should preferably be investigated under flow conditions.

The aim of present study was to evaluate whether certain streptococcal strains interfere with the colonization of cultured epithelial cells by *A. actinomycetemcomitans*, using an *in vitro* model under hydrodynamic conditions.

Material and methods

Epithelial cell culture

HOK-18A cells were cultured as previously described (21). This oral epithelial cell line is derived from normal human oral keratinocyte cells and cells were immortalized by transfecting them with recombinant human papillomavirus type 18 DNA (6). Cells were grown in tissue culture flasks with keratinocyte cell medium (KCM), supplemented with human recombinant epidermal growth factor (5 ng/ml) and bovine pituitary extract (50 μg/ml) (Gibco, Life Technologies Ltd., Paisley, UK), in a 37°C humidified atmosphere containing 5% CO_2. After trypsinization (16) cells were used to seed 24-well tissue culture plates (Iwaki microplate; Scitech, Diu, Japan) containing glass disks (2 mm thick, 7–8 mm in diameter).

Bacterial cell culture

A. actinomycetemcomitans strain ATCC 43718 (also called Y4) was used, a non-autoaggregating smooth laboratory strain of serotype b (7). This serotype is strongly associated with periodontal disease *in vivo* (30).

A. actinomycetemcomitans, *S. sanguinis* KTH-4, *Streptococcus cristatus* CC5A, *Streptococcus salivarius* TOVE and *Streptococcus mitis* BMS were grown on blood agar plates (Blood Agar Base II; Oxoid, Basingstoke, UK), supplemented with 5 mg/ml hemin, 1 mg/ml menadion (Merck, Darmstadt, Germany) and 5% (volume/volume) sterile horse blood (Biotrading, Keerbergen, Belgium). Colonies were picked off and cultured overnight in brain–heart infusion (BHI; Oxoid) at 37°C in 5% CO_2. After harvesting by centrifugation (7970 ***g*** for 10 min), the bacterial pellets were resuspended in fresh medium at a concentration of 1×10^8 colony-forming units (CFU)/ml. The adaptation was based on optical density (OD) measurements at a wavelength of 600 nm.

Prior testing of experimental conditions

A modified Robbins device set-up was used to evaluate bacterial colonization on soft tissue surfaces. Initially, the optimal experimental conditions for both the bacterial and epithelial cells were determined. In a first test, the growth of *A. actinomycetemcomitans* in three media was followed through time by OD measurements at a wavelength of 600 nm. Growth in pure BHI was compared with growth in pure KCM and in a 1 : 1 mixture of both. The same media were also tested for their potential to maintain epithelial cell activity. To measure this factor, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed at different time-points after changing the medium in 24-well tissue culture plates to one of the three test media. MTT is reduced in metabolically active cells to form insoluble purple formazan crystals. For this, the cell medium was removed and monolayers were washed with phosphate-buffered saline (PBS). Each well was filled with 1 ml fresh cell medium and additionally with 100 μl MTT (stock-solution: 5 mg/ml in PBS; Sigma, St Louis, MO). After incubation for 2 h at 37°C in 5% CO_2, supernatant was aspirated and cells were washed with PBS. Cells were incubated for another 15 min with 1 ml dimethylsulfoxide per well to dissolve the formazan crystals. The absorbance was measured with a spectrophotometer at a wavelength of 570 nm. Both tests were repeated three times on independent days.

Streptococcal preinfection

Streptococcal solutions were prepared as described above and 2 h before the start of the exclusion study, cell medium in the 24-well tissue culture plates was replaced by 1 ml of these solutions. The epithelial cells were further incubated at 37°C in 5% CO_2.

Bacterial colonization inhibited in the modified Robbins device

A modified Robbins device (Dentaid, Barcelona, Spain), was used to evaluate the colonization of HOK-18A cells by *A. actinomycetemcomitans* in the presence of streptococcal species versus a control (without streptococcal precolonization) (14, 20).

A suspension of *A. actinomycetemcomitans* was prepared in a 1 : 1 mixture of BHI and KCM and the bacteria were allowed to reach the exponential growth phase before the start of the experiment. Sterile and precolonized epithelial cells on glass disks were mounted in separate rectangular stainless-steel flow chambers of the modified Robbins device. These flow chambers were connected by tubes with a bioreactor, containing a continuous culture of *A. actinomycetemcomitans*. The suspension of *A. actinomycetemcomitans* was pumped through the system at a velocity of 200 μl/min (4). The temperature of the flow chambers and the bioreactor was maintained at 37°C. A gas mixture containing 10% CO_2 was constantly supplied to the system.

Evaluation of bacterial colonization

Samples were removed from the flow chambers at different times after insertion to evaluate the colonization of *A. actinomycetemcomitans* and the epithelial cell number, as previously described (1, 21). Flow was stopped; discs were taken out of the system and placed in glass bottles containing 0.1% Triton-X-100 (Sigma) in PBS. Each bottle was vortexed vigorously for 1 min before 10-fold dilutions in PBS were made (10). Suspensions were plated on agar plates and the number of CFU was determined after incubation for 3 days. Additionally, part of these solutions was used to perform DNA extraction according to the manufacturer's instructions (Instagene Matrix; Bio-Rad, Hercules, CA). A quantitative polymerase chain reaction (QPCR) assay for *A. actinomycetemcomitans* enabled the determination of their total number, both dead and vital (1, 20). Another QPCR assay was performed to estimate the number of epithelial cells present on the samples (21, 29). Quantification of the epithelial cell number was performed using a single copy gene of the human epithelial cells, the human β-defensin-1 gene (11). The *Taq*Man primers and probe sequences, the composition of the reaction mixture and the reaction conditions were based on those described previously (29). Quantification relied on plasmid standard curves, created with known quantities of plasmid DNA containing the target DNA sequences. To construct the plasmids, template DNA was first prepared using Instagene matrix. Next, the primer–probe regions were amplified with PCR. The amplicons were purified and ligated into the pGEM-T Easy vector system (Promega, Madison, WI) according to the instructions of the manufacturer. The plasmids were cloned in *Escherichia coli* DH5α.

The QPCR assays allowed us to express the bacterial colonization as CFU per epithelial cell to avoid artifacts caused by epithelial cell numbers.

The colonization experiment was repeated eight times for each streptococcal strain tested, on randomly chosen days and with fresh bacterial cultures.

Statistical analysis

Data from the previous testing experiments were analysed using analysis of variance. The culture and QPCR data from the exclusion assays were log-transformed before analysis. To take into account the number of epithelial cells present, ratios were taken for each sample of the bacterial number versus the epithelial cell number. A log-transformation was performed on the ratios obtained. A linear mixed model was fitted, with streptococcal strain and time as crossed fixed factors and experiment as the random factor. Pairwise comparisons were calculated and for each comparison a correction for simultaneous hypotheses was performed according to Bretz et al. (2).

Results

Evaluation of experimental conditions

Bacterial and cell growth media are very different in terms of their composition. Therefore they will only ensure an optimal viability and growth of the biological cell type for which they are intended. In the following experiment both the epithelial cells and bacteria need to be in their optimal metabolic condition and able to grow so we needed to develop a modified growth medium that met both the epithelial cell and bacterial metabolic requirements.

The metabolic activity of the epithelial cells, which is correlated with the vitality of the cells, was followed through time to evaluate three media. The absorbances, obtained with the MTT assay, are depicted in Fig. 1A. When the epithelial cells were incubated with BHI, a clear decrease in metabolic activity over time was noticed. A medium containing KCM and BHI in a 1 : 1 v/v ratio (mix medium) and pure KCM ensured the metabolic epithelial activity. The differences in metabolic epithelial cell activity between the KCM and BHI became statistically significant ($P < 0.05$) after 2 h. The decrease in cellular activity compared with baseline (0 h) was statistically significant ($P < 0.05$) for BHI after 3 h. No statistically significant differences were detected between the metabolic activity of epithelial cells incubated in KCM and the mix medium.

The growth of *A. actinomycetemcomitans* in the three media over time is presented in Fig. 1B. The bacteria grew well in the mix medium, even better than in BHI. These differences were statistically significant ($P < 0.05$) after 3 h. In pure KCM almost no growth of *A. actinomycetemcomitans* occurred. KCM started to give statistically significantly ($P < 0.05$) lower results compared with mix medium after 2 h and compared with BHI after 3 h. The average absorbance increased between 0 and 6 h with a factor of 2.6 for BHI, 3.8 for the mix medium and 1.3 for pure KCM. In BHI and in the mix medium a statistically significant ($P < 0.05$) increase in absorbance compared with baseline (0 h) was detected after 2 h. In pure KCM no statistically significant ($P > 0.05$) increase in absorbance compared with baseline (0 h) was detected at any time.

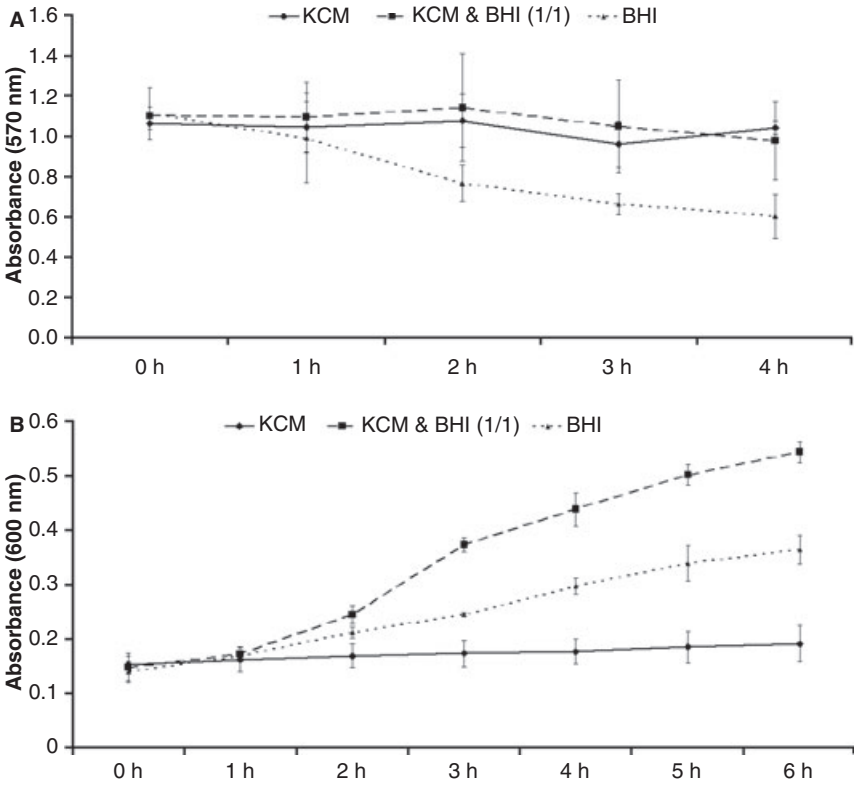

Fig. 1. Culture media tested on epithelial cell activity (A) and *Aggregatibacter actinomycetemcomitans* proliferation in time (B). Three media were tested: keratinocyte cell medium (KCM), a 1 : 1 mixture of keratinocyte cell medium and brain–heart infusion (BHI) broth, and BHI broth. (A), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were used to detect changes in epithelial cell activity in the presence of the three media during 4 h. Absorbances were measured at a wavelength of 570 nm and were expressed as a function of the time. (B) The growth of *A. actinomycetemcomitans* during 6 h in the three test media, expressed as the optical density measured at a wavelength of 600 nm as a function of the time. Bars in both graphs represent standard errors of the mean.

Microbial interference with *A. actinomycetemcomitans* colonization of soft tissues

The number of epithelial cells on the samples showed no statistically significant ($P > 0.05$) changes over the 3-h time period of the colonization experiment. There were also no significant differences in the epithelial cell numbers of the *S. salivarius*, *S. mitis* and *S. cristatus* precolonized monolayer versus the not precolonized control (Fig. 2). However, epithelial cell numbers were statistically significantly ($P < 0.05$) higher on the *S. sanguinis*-precolonized monolayer and the not precolonized control monolayer at the first measurement of the experiment (0.5 h). After 0.5 h no significant differences were observed.

To assess the streptococcal exclusion effect on *A. actinomycetemcomitans* colonization of epithelial cells, this shift in epithelial cell numbers was taken into account. *A. actinomycetemcomitans* colonization (determined with both microbial culturing and QPCR) was therefore expressed as the ratio of the number *A. actinomycetemcomitans* versus the number of epithelial cells in each sample.

Comparison of the ratios based on the culture results (number of viable *A. actinomycetemcomitans*; Fig. 3A) and based on the QPCR results (*A. actinomycetemcomitans*

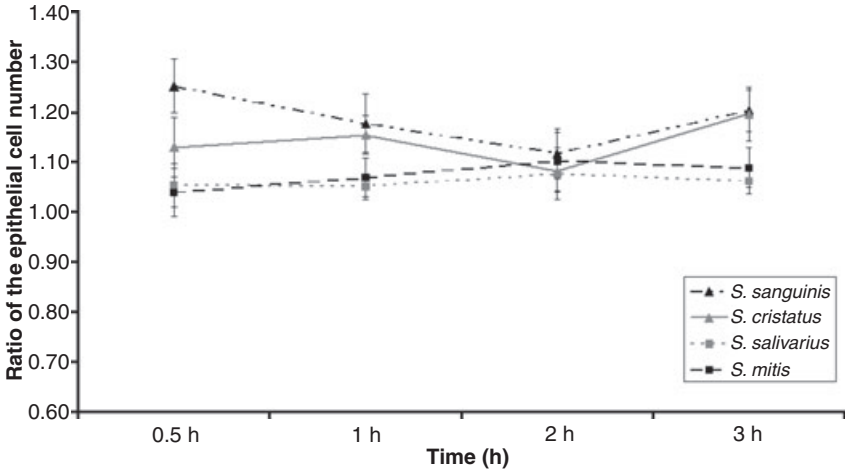

Fig. 2. Epithelial cell number after infection of precolonized and not precolonized monolayers with *Aggregatibacter actinomycetemcomitans*. Epithelial cells were precolonized by *Streptococcus sanguinis*, *Streptococcus cristatus*, *Streptococcus salivarius* or *Streptococcus mitis*. Non-precolonized cells were used as controls. Data were expressed as the ratio of the epithelial cell numbers of precolonized versus not precolonized monolayers (1) as a function of time after infection with *A. actinomycetemcomitans*. Bars represent standard errors of the mean.

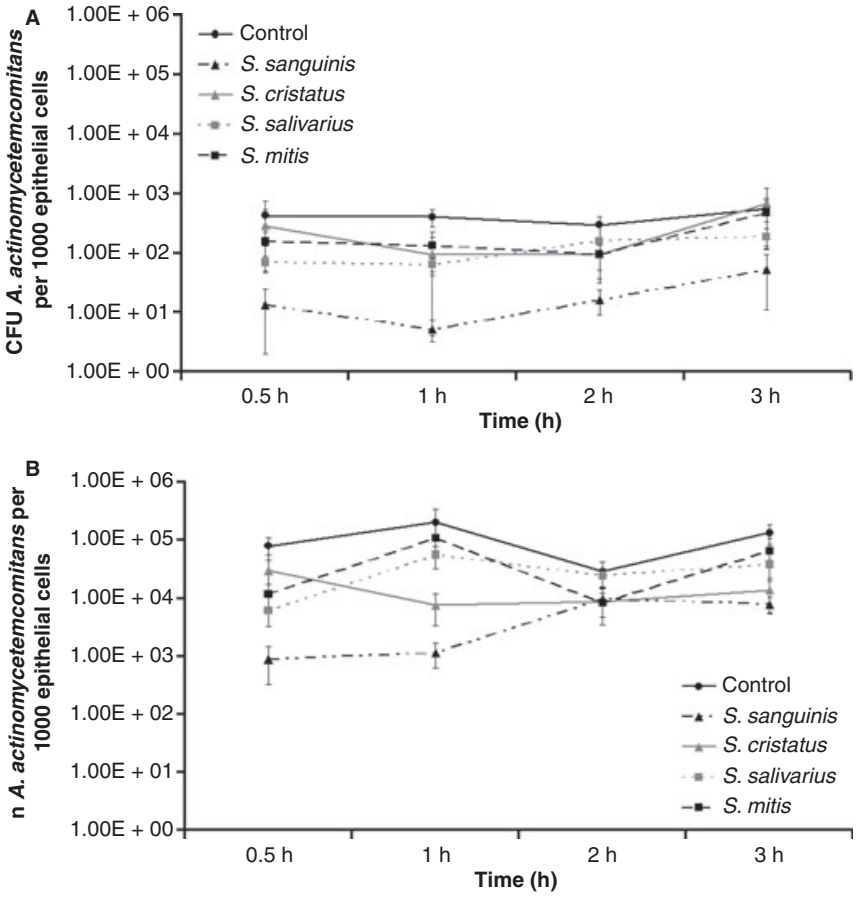

Fig. 3. Aggregatibacter actinomycetemcomitans colonization expressed versus the epithelial cell number. Colony-forming units (A) and total bacterial numbers (B) for *A. actinomycetemcomitans* were expressed per 1000 epithelial cells. Epithelial cells were precolonized with *Streptococcus salivarius*, *Streptococcus mitis*, *Streptococcus sanguinis* and *Streptococcus cristatus* and subsequently infected with *A. actinomycetemcomitans* for 3 h. Controls were clean, not precolonized cells. Bars represent standard errors of the mean.

biomass; Fig. 3B) showed interference with *A. actinomycetemcomitans* colonization if the epithelial cells were precolonized with the four streptococcal species.

The most pronounced reduction ($P < 0.05$) in *A. actinomycetemcomitans* colonization of epithelial cells was caused by *S. sanguinis.* Microbial culturing data and QPCR data showed respectively a 90.5 and 94.1% reduction in *A. actinomycetemcomitans* colonization on *S. sanguinis*-precolonized epithelial cells, compared with *A. actinomycetemcomitans* colonization on not precolonized monolayers, 3 h after the start of the colonization experiment.

The average reduction in *A. actinomycetemcomitans* colonization of epithelial cells over time caused by precolonization with *S. cristatus*, *S. salivarius* and *S. mitis* was 20.3% for the number of viable colonized *A. actinomycetemcomitans* bacteria, and 71.3% for the colonized *A. actinomycetemcomitans* biomass, 3 h after the start of the colonization experiment.

Discussion

In the present study a modified Robbins device was used to evaluate the effect of preinfecting epithelial cells with certain streptococcal species on the colonization of the periodontopathogen *A. actinomycetemcomitans* under hydrodynamic conditions. The optimal experimental conditions were determined by prior testing. The test medium assured a good retention of cellular activity and growth of *A. actinomycetemcomitans*. To avoid artifacts caused by cell number differences, bacterial colonization was expressed as the bacterial number per epithelial cell. According to these relative culture and QPCR results, *S. salivarius*, *S. mitis*, *S. cristatus* and *S. sanguinis* had inhibiting effects on the colonization of *A. actinomycetemcomitans*.

The bacterial numbers for *A. actinomycetemcomitans* determined with QPCR were an average of 2–3 log higher than the conventional culture counts. There are two main explanations for this phenomenon. First of all, bacterial culturing is a technique that is sensitive to aggregation. The detected bacterial number can therefore be lower than the real amount of bacteria present. QPCR, however, is insensitive to aggregation because it is based on detection of a specific gene sequence. Another reason for the higher QPCR values is that the QPCR technique counts all the cells, both dead and vital.

Culture only recovers the cultivatable, viable ones.

To study bacterial colonization and test interference over time, experimental models are needed that mimic the real life situation. Hydrodynamic conditions, present in the *in vivo* ecological niche of the bacteria, were taken into account in this study because these conditions might produce a clearance effect and shear forces with significant impact on the results. The modified Robbins device used in the present study is a flow cell with the important advantage that the influence of multiple species can be tested in parallel (10). Different samples can be placed in the flow chambers and removed at different time-points for further analysis. In the current study it was shown that this set-up can be used to evaluate the interference during bacterial colonization on soft tissue surfaces under hydrodynamic conditions, although follow-up of the soft tissue surface is recommended.

Beneficial bacteria could be applied in 'replacement therapy' to inhibit the colonization process of periodontopathogens on the host surfaces by forming a protective biofilm (25). Probiotics are widely known for their positive influences on the gastrointestinal tract (13), but probiotics are also used in the treatment and prevention of other pathologies (9, 17). The use of 'health-promoting' bacteria for therapeutic purposes in the oral cavity is still a rather new and unexplored concept (18, 26).

S. cristatus and *S. mitis* had an inhibiting effect on *A. actinomycetemcomitans* colonization of soft surfaces under flow conditions, but showed no exclusion effect in a previously performed static study on epithelial cells (25). The main difference between the present dynamic study and the previous static study was the introduction of fluid flow. The forces and clearance effect caused by these fluid flow conditions might be responsible for discrepancies between the microbial culture data of both studies. It could be hypothesized that the clearance effect would lead to less pronounced exclusion effects of the streptococcal species under hydrodynamic conditions because the responsible factor might be washed away by the continuous medium flow. However, the soft tissue experiments conducted in the modified Robbins device show, on the contrary, more pronounced reductions in *A. actinomycetemcomitans* colonization by all four streptococcal species compared with the static-assay results. The effect of *S. sanguinis* was for example increased by approximately 20%. These rather unexpected generally improved inhibitory effects might be explained by an increased sensitivity towards steric hindrance under fluid flow conditions. So, although the major conclusions between the static and dynamic studies were similar, some significant differences in the results could be noticed upon introduction of hydrodynamic conditions.

Based on present and previous studies, *S. sanguinis* appeared to be the best inhibitor of subsequent colonization by *A. actinomycetemcomitans* on hard and soft surfaces, under hydrodynamic and static conditions (20, 25). The exclusion effect of the streptococci seemed to be more pronounced on the soft tissue surfaces in the present study, compared with the hard surfaces in a previous study (20). Different possible explanations can be proposed. (i) One of the mechanisms behind the exclusion effect might be direct competition for the same epithelial cell receptor. On hard surfaces the adhesion of *A. actinomycetemcomitans* is known to occur less specifically (3). This might explain why exclusion occurs better on the soft tissue surfaces. (ii) Changes in the epithelial cell membrane, induced directly by the streptococci, could also partly cause the exclusion effect on soft tissue surfaces (25). This phenomenon cannot occur on the hard surfaces. This would also clarify the higher exclusion efficiency of the streptococci on the soft tissue surfaces. (iii) The exclusion effect could be partly caused by factors produced (and secreted) by the epithelial cells under the influence of the streptococcal species. These factors would be absent in the hard surface assays. This so-called 'conditioning of the environment' by the epithelial cells hypothesis was previously described by Teughels et al. (25). They concluded that *S. sanguinis* could trigger the epithelial cells to secrete factors that lower the *A. actinomycetemcomitans* growth rate and change *A. actinomycetemcomitans* colonization of epithelial cells. *S. mitis* could trigger epithelial cells to condition cell medium so that it inhibits *A. actinomycetemcomitans* colonization; *S. salivarius* did not trigger the epithelial cells to condition the medium, but conditions it by itself (25).

Hence, the exact mechanism behind the exclusion effect is not known. Some other possible explanations for the exclusion effect on both hard and soft tissue surfaces are: the production of bacteriocines, bacteriocine-like inhibitory substances and/or hydrogen peroxide by the streptococcal species. It is known that *S. sanguinis* produces a bacteriocine (sanguicine) and hydrogen peroxide (5). *S. salivarius* inhibits the emergence of *Streptococcus mutans in vivo* (24). *S. mitis* can release biosurfactants as an indirect defense mechanism against other colonizing strains on the same substratum (27). It is also possible that the streptococci produce some other (unknown) substance that will either work directly on the growth or viability of *A. actinomycetemcomitans*, or will modify and condition the environment in a way that affects the colonization of *A. actinomycetemcomitans* (25).

In a very recent study van Hoogmoed et al. (28) evaluated the capacity of *S. sanguinis*, *S. crista*, *S. salivarius*, *S. mitis*, *Actinomyces naeslundii* and *Haemophilus parainfluenza* in reducing the adhesion of periodontopathogens using a parallel plate flow chamber. The pathogens used were *A. actinomycetemcomitans*, *P. gingivalis* and *Prevotella intermedia*. Adhesion of the pathogens was studied for 2 h on the polymethylmethacrylate bottom plate of a flow chamber. Evaluation was performed with *in situ* visualization using phase-contrast microscopy. The main results from this study were that *A. naeslundii* and *S. mitis* caused the strongest significant suppression of adhesion. The highest reduction was seen for *P. gingivalis*. The best inhibitors of *A. actinomycetemcomitans* were *S. salivarius* and *A. naeslundii* (28). The discrepancies with our results might be the result of differences in the experimental set-up. A so-called 'dot assay' was used by these researchers, whereby 25-μl suspension droplets were placed on the hard surface bottom plate of the flow chamber for 30 min to allow adhesion of the potentially beneficial bacteria (28). In our study beneficial species were allowed to interact with the soft surface for 2 h before the exclusion assay. According to these authors, the differences in effects suggest that interference mechanisms other than or besides physical blocking were involved (28). Although there were some differences in the order of efficiency, probably because of heterogeneity in the experimental set-up, this study agrees with our research in that it has been affirmed that replacement therapy could be a therapeutic alternative towards periodontal pathogens. Certain antagonistic strains could be used to control the adhesion of pathogens and prevent infection.

QPCR results revealed no major changes in epithelial cell numbers with

time. This ensures that the test model allows a good retention of the cellular monolayers. The number of epithelial cells after precolonization with *S. sanguinis* was temporarily significantly higher than in the not precolonized samples, infected with only *A. actinomycetemcomitans*. This difference in epithelial cell numbers might be interpreted as a protection of the streptococci towards the cytotoxic effect of *A. actinomycetemcomitans*. The *A. actinomycetemcomitans* strain used in the present study (ATCC 43718 = Y4) is known to produce a cytolethal distending toxin, a cytotoxic factor that is secreted into the bacterial culture supernatant (15). This toxin causes necrosis of the epithelial cells and fibroblasts (22). Studies have shown that 72 h after infection of KB cells *in vitro* with *A. actinomycetemcomitans* strain Y4 only 10–20% of the epithelial cells show cytotoxic effects. This amount increases up to 50% after 120 h (8). It seems therefore rather unlikely that these cytotoxic effects would have occurred 0.5 h after the start of *A. actinomycetemcomitans* infection. If cytotoxicity as a result of *A. actinomycetemcomitans* infection took place here, a significant decrease in the number of epithelial cells with time should have occurred after monoinfection with *A. actinomycetemcomitans* and this was not the case. The *S. sanguinis* seemed to be able to offer, at least temporarily, some protection against epithelial cell detachment. The exact mechanism remains obscure, however, and it can also not be ruled out that these cell number differences were only caused by coincidence. Anyway, it is important to correct for these differences in cell numbers to avoid artifacts in the data interpretation.

The results obtained showed that the streptococcal species tested, mainly *S. sanguinis*, exert an exclusion effect towards *A. actinomycetemcomitans* colonization on soft tissue surfaces under flow conditions. The exact nature of these interactions warrants further investigation. These data support the idea that bacterial interactions could possibly be used as a new treatment approach for periodontal disease. The new treatment strategy could rely on the creation of a healthy microbiota in the oral cavity to interfere with the colonization of periodontopathogens.

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