

Establishment of an oral infection model resembling the periodontal pocket in a perfusion bioreactor system

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Periodontal infection involves a complex interplay between oral biofilms, gingival tissues and cells of the immune system in a dynamic microenvironment. A humanized in vitro model that reduces the need for experimental animal models, while recapitulating key biological events in a periodontal pocket, would constitute a technical advancement in the study of periodontal disease. The aim of this study was to use a dynamic perfusion bioreactor in order to develop a gingival epithelial-fibroblast-monocyte organotypic co-culture on collagen sponges. An 11 species subgingival biofilm was used to challenge the generated tissue in the bioreactor for a period of 24 h. The histological and scanning electron microscopy analysis displayed an epithelial-like layer on the surface of the collagen sponge, supported by the underlying ingrowth of gingival fibroblasts, while monocytic cells were also found within the sponge mass. Bacterial quantification of the biofilm showed that in the presence of the organotypic tissue, the growth of selected biofilm species, especially *Campylobacter rectus*, *Actinomyces oris*, *Streptococcus anginosus*, *Veillonella dispar*, and *Porphyromonas gingivalis*, was suppressed, indicating a potential antimicrobial effect by the tissue. Multiplex immunoassay analysis of cytokine secretion showed that interleukin (IL)-1 β , IL-2, IL-4, and tumor necrosis factor (TNF)- α levels in cell culture supernatants were significantly up-regulated in presence of the biofilm, indicating a positive inflammatory response of the organotypic tissue to the biofilm challenge. In conclusion, this novel host-biofilm interaction organotypic model might resemble the periodontal pocket and have an important impact on the study of periodontal infections, by minimizing the need for the use of experimental animal models.

Introduction

The two major forms of periodontal diseases are gingivitis and periodontitis. Approximately 80% of the world population exhibit gingivitis,¹ while 15% may exhibit severe destructive periodontitis.² Periodontitis is the primary cause of adult tooth loss due to its destructive effect on the periodontal connective tissues and underlying alveolar bone.³ Besides, recent evidence shows that the effects of periodontal infections may well expand beyond the oral cavity, to be implicated in systemic diseases, such as diabetes mellitus and cardiovascular diseases.^{4,5}

The causation of these infections involves the complex interaction between biofilm communities forming on the tooth surface, and the neighboring host periodontal tissues.⁶ Biofilms are polymicrobial communities embedded in a dense extracellular matrix

produced by the different constituent species.⁶ More than 700 species⁷⁻⁹ have already identified in the oral cavity that may colonize the biofilms, but the actual number could be even higher, due to the limitations in detection by the contemporary technologies.¹⁰ As constituents of the biofilm, bacteria can better withstand environmental stresses and receive metabolic benefits from each other.⁶ The health-associated biofilms do not trigger a strong inflammatory response. In fact, some studies indicate that these biofilms actually benefit gingival health, by locally boosting the immune systems.⁹ However, a biofilm could start to trigger inflammation when the composition of bacterial community shifts to a more pathogenic one.^{11,12}

In response to the biofilm accumulation, the host tissue produces an inflammatory response for the recruitment of immune cells (e.g. polymorphonuclear leukocytes, monocytes),³ and

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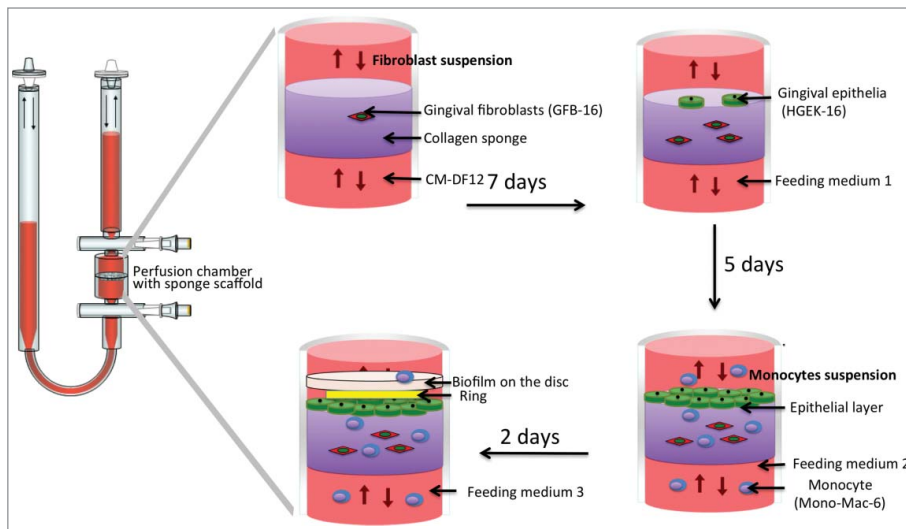


Figure 1. Schematic representation of the procedure used to establish the biofilm-3D gingival organotypic tissue co-culture model.

secretes a number of small compounds of the immune system (e.g. antibodies, cytokines and prostaglandins). If the inflammation tends to become chronic and the biofilm fails to be eliminated, the network of inflammatory molecules that has been established can cause connective tissue damage and bone destruction.^{3,13} In some severe periodontitis cases, this may eventually lead to tooth loss if the inflammation remains unresolved.¹⁴

Several models have been used to understand mechanisms underlying periodontal infections, however, none of them appears to be reliable and reproducible. Experimental animals models of oral infection cannot efficiently represent human oral pathogenic bacteria,^{15,16} whereas human experimental studies may answer questions regarding the initiation of these diseases,¹⁷ but difficult to identify mechanisms that convert protective inflammation to tissue destructive lesion, due to ethical considerations. Most studies using in vitro models have only employed single oral bacterial species to challenge 2-dimensional monolayer cells,^{18,19} or cells in suspension,²⁰⁻²² despite that periodontal infections are biofilm-related.²³ Moreover, monolayer cell culture systems do not adequately mimic the morphological and functional features of primary gingival tissues.²⁴

Previous studies have shown 3D organotypic culture systems to mimic the in vivo situation better than 2D monolayer cultures.^{25,26} It is known that epithelial cells differentiate to their nature state in primary tissue with the support of underlying fibroblasts in the connective tissue.^{25,27} Therefore, 3D models using both epithelial cells and fibroblasts are preferable for the study of gingival tissues.²⁸⁻³² Besides, during chronic periodontal inflammation, it is possible that part of the epithelial layer of the tissue is degraded and the underlying fibroblast-comprised connective tissue may directly confront the oral biofilm. Indeed, fibroblasts are also actively participating in bacterially-induced inflammation,^{33,34} and there are studies available that investigate their interaction with biofilms in vitro.³⁵⁻³⁷

It is technically very challenging to reconstitute multi-tissue structures, such as gingiva, in in vitro cultures. Bioreactors, including perfusion systems, have been developed and used for in vitro modeling of various other infections including the lung,³⁸ the intestine,^{39,40} and the colon.^{41,42} Such a system could mimic the natural environment at local sites (e.g., temperature, atmosphere, shear stress), allowing for the study of biofilm-related mucosal disease under more realistic conditions. Yet, such a system has not been so far implemented in the field of periodontal infections. Apart from structural cells (i.e. fibroblasts and epithelial cells), cells of the immune system, such as monocytes can be readily incorporated into a perfusion bioreactor system, for the study of the biofilm challenge in an environment simulating the established periodontal pocket.

The aim of this study was to develop a gingival organotypic tissue consisting of gingival epithelial cells, fibroblasts and monocytic cells, in a dynamic environment, which was achieved with the use of a perfusion bioreactor system. An 11-oral (subgingival) species biofilm grown on hydroxyapatite discs, which was established in our laboratory based on the previous models,^{36,43-46} provides a model closer to the in vivo situation than single or few bacterial species. This was used to challenge the generated organotypic gingival tissue within the bioreactor, in a low oxygen environment, thus validating the usability of this organotypic tissue. It is anticipated that this novel dynamic tissue-biofilm interaction model may mimic the periodontal pocket environment and thus provide a more accurate in vitro experimental platform for the study of periodontal infections.

Results

Histological analysis of the organotypic tissue on the collagen sponge

The GFB-16, HGEK-16, and Mono-Mac-6 cells were seeded on the collagen sponges under the U-CUP perfusion bioreactor to construct 3D in vitro gingival tissue as described in materials and methods. The biofilm challenge was later introduced to the system for 24 h, to mimic periodontal infection. The morphology of the 3D model was evaluated by Masson's Trichrome stain (Fig. 2). In the control group whereby the biofilm was absent (Fig. 2a), there was a consistent epithelial-like layer of cells formed on the surface of the collagen, while inside the collagen structure, cells with long protrusions spread along the collagen fibers were observed. In the presence of biofilms (Fig. 2b), the epithelial-like layer was disrupted, with the appearance of many rounded big cells next to the collagen surface, that were not detectable in the control group.

Scanning electron microscopy (SEM) analysis of the organotypic tissue surface

SEM was used to analyze the structure of the collagen sponge surfaces in contact with the biofilms. As seen from this analysis, the collagen sponge that served as a scaffold displayed a regular mesh of layers (Fig. 3a). When seeded onto the collagen sponge, cells filled most gaps between collagen fibers and formed a dense structure (Fig. 3b). In the collagen sponge, on which the biofilm was seeded (in the absence of host cells), clumps of predominately coccoid bacteria were observed in conjunction to the collagen fibers, and all over the mass of the sponge (Fig. 3c; Fig. S1). Such clumps of bacterial colonies were not found when the biofilms were co-cultured in the presence of the 3D gingival tissue, despite the presence of many large, monocyte-like cells (Fig 3d), rather than the layered cells that were previously observed when the cells were seeded alone (Fig. 3b).

Quantification of biofilms

After 24 h in co-culture with the organotypic gingival tissue, the bacterial composition of the biofilm was evaluated and compared to that of the biofilm in culture with the collagen sponge alone (Fig. 4). Significantly different numbers between the two groups were detected for *C. rectus*, *A. oris*, *S. anginosus*, *V. dispar*, and *P. gingivalis* (33.28-fold, $P = 0.0002$; 11.09-fold, $P = 0.0067$; 16.65-fold, $P = 0.0036$; 17.46-fold, $P = 0.0266$; and 19.28-fold, $P = 0.0014$; respectively). Other differences did not prove to be statistically significant, hence the numbers of the remaining species were not affected.

Quantification of cytokines secretion

Finally, the level of cytokines in the culture medium was investigated by the use of a 10-plex immunoassay platform (Fig. 5). After 24 h of challenge, the biofilm caused a significant increase in the production of IL-1 β , IL-2, IL-4, and TFN- α by the organotypic gingival tissue, compared to the unchallenged control tissue alone (56.01-fold, $P = 0.0011$; 6.94-fold, $P < 0.0001$; 1.70-fold, $P < 0.0001$; and 45.63-fold, $P = 0.0006$; respectively). This finding confirms the inflammatory-

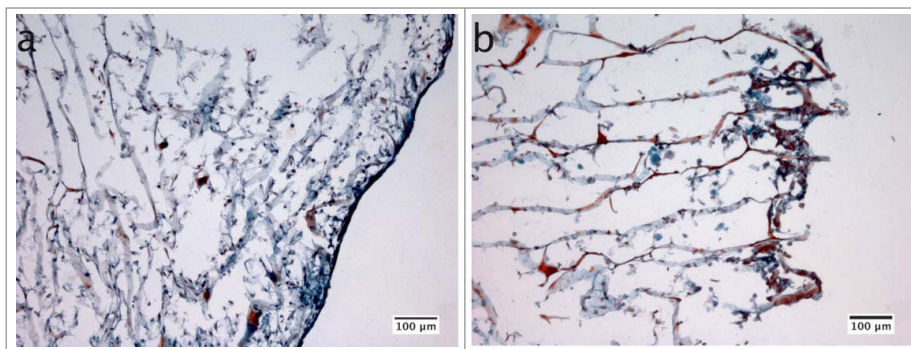


Figure 2. Masson's trichrome staining used for histological analysis of the collagen sponges, in which the 3D gingival tissue had been reconstructed in vitro. The tissue was co-cultured for 24 h with hydroxyapatite discs without (a) or with the biofilm (b).

inducing capacity of the biofilm on the organotypic gingival tissue.

Discussion

In this study, we developed an organotypic co-culture system for the study of interactions between gingival tissues and subgingival biofilms in a U-CUP perfusion bioreactor device. The model shows compatible histological features to a periodontal

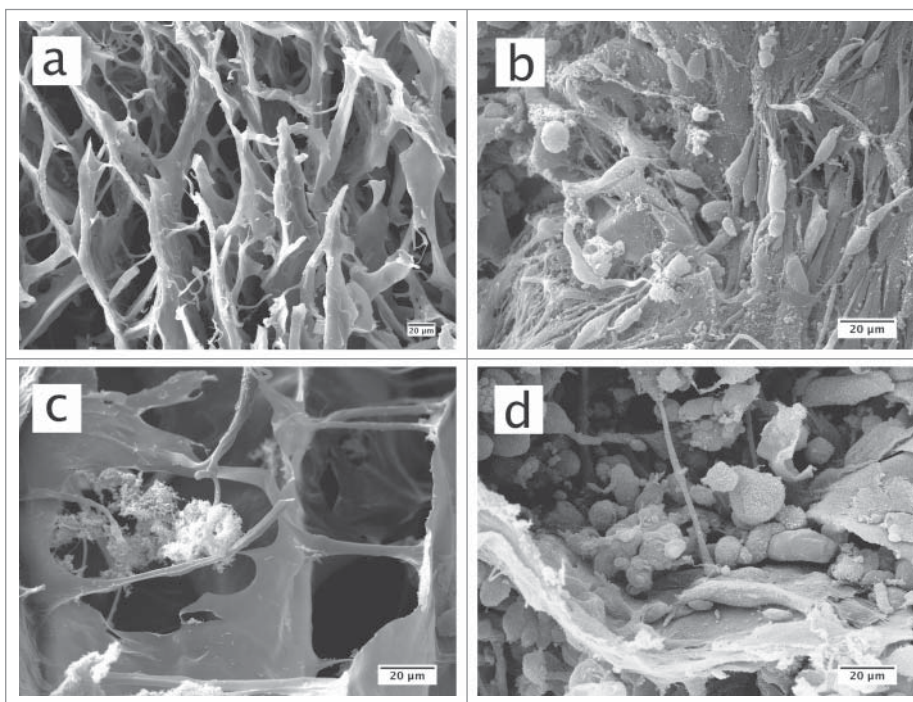


Figure 3. SEM images of original collagen sponge structures (a), collagen sponges on which the 3D in vitro gingival tissue had been reconstructed (b), collagen sponges in the presence of biofilms only (c), or collagen sponges on which the 3D in vitro gingival tissue had been reconstructed and challenged with the biofilms for 24 h (d).

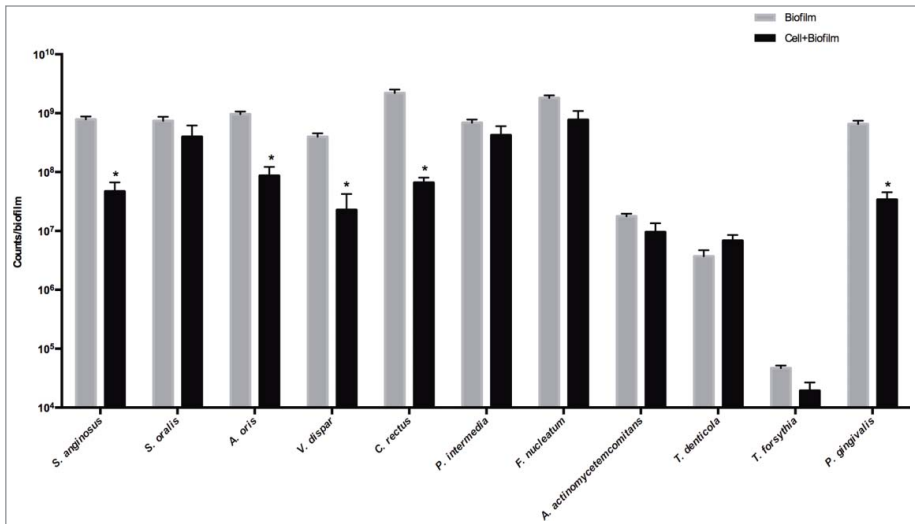


Figure 4. Quantification of bacterial numbers in biofilms. The biofilms were either cultured on blank collagen sponges (biofilm), or co-cultured with the 3D in vitro gingival tissue for 24 h (biofilm + cell). These values represent mean values of triplicate experiments \pm standard deviation (SD). The results are presented on a logarithmic scale. Asterisk (*) represents the significance of differences ($P \leq 0.05$) between the 2 groups.

pocket in vivo and utilizes a biofilm in order to challenge a multi-layered epithelial-fibroblast tissue, constructed using previously established immortalized human gingival cells,³² and enriched further with monocytic cells.

As first line of the host defense, gingival tissue provides an epithelial layer firmly aligned onto the tooth surface.⁴⁷ Many approaches have been applied to create such an epithelial structure in vitro, for the study of the mechanisms of periodontal diseases. Moreover, the gingival epithelium is supported by the gingival connective tissue,⁴⁷ which consist of fibroblasts that produce a firm collagen network and produce important growth

established over time.

In our model, collagen sponges were used to create such a space while allowing gingival fibroblast and epithelial cells to attach onto them. Thus, a 3-phase seeding protocol was developed, ensuring that an epithelial layering and underlying connective tissue is formed, while maintaining constant flow of culture medium and cells of the immune cell. This system was microbially-challenged with an in vitro generated subgingival biofilm, grown onto hydroxyapatite surface (analogous to the tooth surface). Previous experimental models have already used single bacteria species, including *A. actinomycetemcomitans*, *F. nucleatum*, to challenge an epithelial-fibroblasts co-cultured model.⁵⁵⁻⁵⁷ Earlier works in our group have used this subgingival biofilm model in static co-culture either with gingival fibroblasts³⁶ or with multilayer gingival organotypic epithelium, in order to study various aspects of host tissue-oral biofilm interaction.^{58,59}

factors for epithelial survival and growth.^{33,48} Since Tomakidi et al⁴⁹ developed an oral gingival model constituted epithelial cell with underlying fibroblasts in the collagen gel, many similar models were established for the study of gingival and oral mucosa.^{28-30,50,51} However, one of the main challenges to apply such model in the periodontal infection is the incorporation of immune cells in a dynamic environment of continuous flow. It should be noted that at least 2–5% of the total surface of the gingiva that attach to the tooth (i.e., junctional epithelium) is occupied by intercellular spaces.⁵² This looser structure allows gingival crevicular fluid (GCF) to pass through the periodontal pocket. This carries primarily trans-migrating neutrophilic granulocytes and infiltrating mononuclear cells, like macrophages and lymphocytes^{53,54} to the tissue against which the biofilm is

As most subgingival species are strict anaerobes,⁶ they were grown separately under anaerobic conditions, prior to being introduced into the bioreactor environment along with the organotypic tissue. It should be noted that, in vivo, diseased human gingival tissues present in 2% oxygen and are exposed to shear forces by saliva and gingival exudate at flow rate of 0.4–2.0 ml/min.⁶⁰ These microenvironment conditions were also mimicked within the present bioreactor model, demonstrating that both the

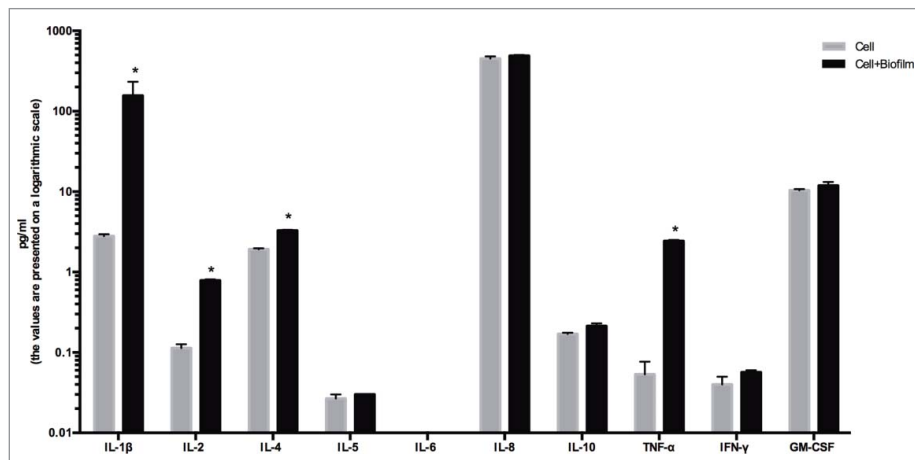


Figure 5. Quantification of cytokine secretion. The 3D in vitro gingival tissue were cultured either in the presence of only pellicle coated hydroxyapatite discs (cell) or co-cultured with biofilm-grown hydroxyapatite discs (biofilm + cell) for 24 h. These values represent mean values of triplicate experiments \pm standard deviation (SD). The results are presented on a logarithmic scale. Asterisk (*) represents the significance of differences ($P \leq 0.05$) between the 2 groups.

organotypic tissue and the bacterial biofilm are able to survive, co-exist and interact during the given experimental period of 24 h. The numbers of some bacterial species (namely *S. anginosus*, *A. oris*, *V. dispar*, *C. rectus* and *P. gingivalis*) were suppressed in the presence of the organotypic tissue, compared to the collagen sponge alone. Accordingly, bacterial aggregates were sparsely detectable on cells-seeded collagen surfaces. This potentially indicates the antimicrobial capacity of the tissue cells and/or the involvement of the immune cells in controlling bacterial colonization in the present experimental model.

To further evaluate the *in vivo* relevance of this model, selected secreted cytokines were measured by multiplex immunoassay technology. We detected the up-regulation of the pro-inflammatory cytokines IL-1 β , IL-2, IL-6, and TNF- α on the secreted bioreactor medium (analogous to GCF), after 24 h of challenge of the tissue with the biofilm. Indeed, several studies have demonstrated that patients with periodontitis also display higher concentrations of IL-1 β ,^{61,62} IL-2,⁶¹ IL-6,^{63,64} and TNF- α ⁶² in GCF, compared to healthy gingiva. The regulation of cytokines or chemokines in gingival tissues is a complex process that involves many cells of the immune system,^{20,65-67} or structural tissue cells.^{68,69} To further enhance this model, additional elements could be introduced into this system, such as neutrophils and B- or T-lymphocytes, following the same protocol as with the Mono-Mac-6 cell lines.

In clinical studies,⁷⁰ increasing number of *T. forsythia*, *P. gingivalis*, *P. intermedia*, and *F. nucleatum* correlated with an up-regulation of IL-1 β , while increasing number of *S. oralis*, and *S. anginosus* correlated with up-regulation the TNF- α , which may be in line with our observations in this *in vitro* experimental system. With regards to *in vitro* studies, the effects of periodontal bacteria are more representative when part of biofilm communities. Hence, the comparison of the effects of 2 biofilms with slightly altered bacterial composition can be useful for evaluating the relative involvement of different species in tissue cytokine responses.⁵⁹

In conclusion, we have developed a novel model system to resemble the periodontal pocket, which allows for the simultaneous study of the interaction between oral biofilms, gingival tissues and cells of the immune system, in a biologically relevant environment. To our knowledge, this is the first model that utilized a perfusion bioreactor to study periodontal infection *in vitro*, and its establishment could also have an impact in the study of other biofilm-related diseases. Moreover, this *in vitro* system may reduce the need for usage of experimental animal models in periodontal research.

Materials and Methods

Cell culture

The development of the present 3D cell culture model required a combination of gingival epithelial keratinocytes, gingival fibroblasts and monocytic cells. Immortalized human gingival epithelial keratinocytes (HGEK-16) and immortalized human gingival fibroblasts (GFB-16) were established by transducing

E6/E7 oncoproteins from human papillomavirus type 16 (HPV16-E6/E7) to primary cells.³² HGEK-16s were maintained in complete epithelial medium (CM-DKSFM) consisting of defined keratinocyte serum free medium (Gibco, 10744-019), supplemented with 100 U/ml penicillin (Sigma, 15140-122), 100 μ g/ml streptomycin (Sigma, 15140-122), 2 mM L-glutamine (Sigma, G7513), and 0.25 μ g/ml fungizone (Sigma, 15290-018). GFB-16s were maintained in complete fibroblasts medium (CM-DF12) Dulbecco's modified Eagle medium consisting of nutrient mixture F-12 media (Sigma-Aldrich, D8900-10L), supplemented with 1.2 g/ml of Na-Bicarbonate (Sigma-Aldrich, S5761), 10 % PAN Sera FBS special designed for ES cells (FBS, Bioswisstec AG, 2602), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 0.05 μ g/ml fungizone.

The human myelomonocytic cell line Mono-Mac-6 obtained from the German Collection of Microorganisms and Cell Cultures (Mascheroder, Braunschweig, Germany) was maintained in complete myelomonocytic medium (CM-RPMI) consisting of RPMI-glutamax (Gibco, 72400-021), supplemented with 10% fetal bovine serum, 1% non-essential amino acids (Gibco, 11140-035), 1% sodium pyruvate (Gibco, 11360-039) and 9 μ g/ml bovine insulin (Sigma, 8418) 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.05 μ g/ml fungizone.

In vitro biofilm formation

An 11 species biofilm was established based on a previously developed Zürich 10 species subgingival biofilm model.^{43,71} The following strains were used included in this model: *Prevotella intermedia* ATCC 25611T (OMZ 278), *Aggregatibacter actinomycetemcomitans* JP2 (OMZ 295), *Campylobacter rectus* (OMZ 398), *Veillonella dispar* ATCC 17748T (OMZ 493), *Fusobacterium nucleatum* subsp. *nucleatum* (OMZ 598), *Streptococcus oralis* SK248 (OMZ 607), *Treponema denticola* ATCC 35405T (OMZ 661), *Actinomyces oris* (OMZ 745), *Streptococcus anginosus* ATCC 9895 (OMZ 871), *Tannerella forsythia* (OMZ 1047) and *Porphyromonas gingivalis* W50 (OMZ 308). Briefly, 200 μ l of bacterial cell suspensions containing equal densities (OD₅₅₀ = 1.0) of each strain were mixed with 1.6 ml of growth medium consisting 60% saliva,⁷² 10% human serum, 30% mFUM,⁷³ and 0.5% hemin to initiate biofilm formation on hydroxyapatite discs (diameter 13 mm) (Clarkson Chromatography Products, custom made), pre-coated for 4 h with saliva, which was diluted 1:2 in a mixture of 0.9% NaCl and distilled water. A ring that is slightly larger than hydroxyapatite disc was sheathed around the veneer of the disc. The diameter of inner core at one end of the ring is smaller than 13 mm, therefore able to seal the hydroxyapatite disc inside. This end of the ring is around 1 mm longer than the surface of the hydroxyapatite disc, creating a gap between the biofilm surface and the bottom of the well. The discs were incubated for 64 h in anaerobic conditions, with medium renewed at 16 h and 40 h. During the first medium renewal, additional 40 μ l of *T. denticola* (OD₅₅₀ = 1.0) were further added to each well. The discs were dip-washed in saline at 16 h, 20 h, 24 h, 40 h, 44 h, 48 h and 64 h.

Development of the 3D organotypic culture system in bioreactor

The 3D organotypic culture was established using 3D collagen sponge (porcine collagen, type I) scaffolds (Optimaix, Matricel GmbH, O3D304030) cut into a disc shape with 8 mm in diameter and 3 mm thick in a perfusion bioreactor (UCUP, Cellec Biotek AG, www.cellecbiotek.com, UCUP001) based on a previously developed bioreactor system.^{74,75} Briefly, the scaffold was embedded within a pair of provided adaptors and placed inside the bioreactor chamber, in order to constrain the cell/medium suspension passing through its pores, avoiding thus convective flow (Fig. 1). The flow rate was controlled by the PHD Ultra Syringe Pump (included in the U-CUP bioreactor package).

The protocol for the development of the 3D co-culture in the bioreactor is schematically shown in Fig. 1. Initially, 2×10^6 GFB-16 cells suspended in CM-DF12 were perfused through each $\varnothing 8 \times 3$ mm 3D collagen sponge scaffold, at a superficial velocity of 1000 $\mu\text{m}/\text{sec}$ for 15 h, followed by 100 $\mu\text{m}/\text{sec}$, as previously has been demonstrated to maximize the seeding efficiency of porous scaffold materials and allow subsequent tissue development.^{75,76} After 7 days, the bioreactor chambers were opened under sterile hood and 1×10^6 HGEK-16 cells were statically seeded on the top of same 3D collagen sponge in order to form an epithelial layer on its surface. Cells were incubated in feeding medium 1 (CM-DKSFM: CM-DF12 = 1:1) for 3 h to allow cell attachment before initiating again the perfusion at a velocity of 100 $\mu\text{m}/\text{sec}$. After 5 days, 2×10^6 Mono-Mac-6 cells were injected into the bioreactor system and continued the perfusion at a velocity of 100 $\mu\text{m}/\text{sec}$, in feeding medium 2 (CM-DMEM: CM-DF12: CM-RPMI = 1:1:1). After 2 days, the sponge was washed 3 times with feeding medium 3 (antibiotic free CM-DMEM: CM-DF12: CM-RPMI = 1:1:1) to remove any remaining antibiotic, prior to the addition of the biofilm in the system (description in the next section). The removed medium was also retained in order to collect the Mono-Mac-6 cells by centrifugation, and thereafter added back to the system with feeding medium 3. The cultures were fed every 2 d during this process, before being brought in co-culture with the biofilm.

In order to mimic the conditions of periodontal infection, hydroxyapatite discs with or without (control) the biofilms were introduced into the bioreactor chambers. Briefly, the bioreactor chambers were opened under sterile conditions in tissue culture, and the hydroxyapatite discs were placed by press-fit within the provided adaptors, facing the epithelial surface of the generated 3D organotypic tissue, or the collagen sponge alone (control). The chambers were closed again and perfusion flow continued at a flow rate of 135 $\mu\text{m}/\text{sec}$ in feeding medium 3 for additional 24 h. The culture temperature was 37°C and the atmospheric conditions 2% O₂ and 5% CO₂.

Biofilm harvesting and quantification by real-time quantitative polymerase chain reaction (qPCR)

For the evaluation of the bacterial composition of the biofilm after 24 h of co-cultured, the discs were removed from the bioreactor and vigorously vortexed for 3 min with 1 ml 0.9 % NaCl,

and sonicated at 25 W for 5 seconds. The bacterial suspensions were stored at 20°C before being processed for real-time quantitative PCR (qPCR) analysis. The DNA was extracted from bacterial suspensions using the GenElute™ bacterial genomic DNA kit (Sigma-Aldrich, NA2110) following the gram-positive lysis protocol of manufacturer's guideline with expanded lysis steps from 30 min to 1 h (lysozyme/mutanolysin step), and from 10 min to 30 min (proteinase K step). All primers were designed using online NCBI/Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) targeted 16S rRNA gene (Table 1). The qPCR runs were performed using SYBR® Green PCR Master Mix (Life Technologies, 4309155) in a StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Standard curves were generated using DNA extracted from planktonic cultures of each of the 11 species used in biofilm, and the abundances were calculated using the theoretical genome weight of each organism from each strain according to the NCBI database, as previously described.⁴⁴

Quantification of cytokine secretion

Upon completion of the experiments, the flow culture medium was collected from bioreactors, and centrifuged at 1500 rpm for 5 min before being filtered through 0.2 μm pore size, and stored at -80°C for further uses. The Cytokine Human Ultrasensitive 10-Plex Panel (Novex®, ThermoScientific, LHC6004) was used to quantify cytokines in the collected medium, according to the manufacturer's instructions. The following cytokines were tested in this assay: Interleukin (IL)-1 β , IL-2, IL-4, IL-8, IL-5, IL-6, IL-10, Interferon gamma (IFN- γ), Tumor necrosis factor α (TNF- α), and granulocyte-macrophage colony-stimulating factor (GM-CSF) with sensitivities more than 0.05, 0.05, 0.1, 0.05, 0.1, 0.5, 0.05, 0.1, 0.5, and 0.1 pg/ml, respectively. These cytokines were detected with premixed antibody beads, read on a Luminex®200 system, and analyzed by xPONENT® software (ThermoScientific).

Harvesting of organotypic culture and histological analysis by Masson's Trichrome staining

The collagen sponges containing the generated 3D organotypic gingival tissue were removed from the bioreactor once the experiment was completed. They were then fixed with 3% paraformaldehyde in 2% sucrose/PBS buffer for 2 h, washed 3 \times 20 min in PBS, dehydrated in 15% sucrose/PBS for 2 h, and kept in 30% sucrose/PBS overnight at 4°C.

Slices of harvested sponges were introduced into aluminum foil molds filled with 5% gelatin and 5% sucrose solution, before snap frozen in cold 2-methylbutane (around -40°C by dry ice). The frozen blocks were sectioned into 16 μm and transferred to Superfrost® Plus slides (ThermoScientific, 10143352). The slides were dried in room temperature overnight. They were stained with Weigert's iron hematoxylin (Sigma-Aldrich, 03979) for 20 sec, washed in tap water for 2 min, and rinsed in distilled water, stained with solution A (0.5 g acid fuchsin (Sigma-Aldrich, 857343), 0.5 g ponceau syldine (Sigma-Aldrich, 81465), 1 ml glacial acetic acid (Sigma-Aldrich, 537020), and 99 ml distilled

Table 1 Primer sequences and properties

Organism	Sequence (5' → 3')	Strand on template	Size of amplicon (bases)	T _m (°C)	Ref #
Streptococcus anginosus	ACCAGGTCTTGACATCCCGATGCTA	+	76	59.25	44
	CCATGCACCACCTGTCACCGA	-		59.04	
Streptococcus oralis	ACCAGGTCTTGACATCCCTCTGACC	+	70	59.42	44
	ACCACCTGTCACCTCTGTCCCG	-		59.85	
Actinomyces oris	GCCTGTCCTTTGTGGGTGGG	+	71	59.57	44
	GCGGCTGCTGGCAGTAGTT	-		60.32	
Veillonella dispar	CCCGGGCCTTGACACACCG	+	62	59.7	44
	CCCACCGGCTTTGGGCACTT	-		59.83	
Fusobacterium nucleatum	CGCCCGTCACACCAGAGA	+	75	59.04	44
	ACACCTCGGAACATCCCTCCTTAC	-		59.48	
Campylobacter rectus	TCACCGCCCGTCACACCATG	+	57	59.35	44
	CCGGTTTGGTATTTGGGCTTCGAGT	-		59.5	
Prevotella intermedia	GCGTGCAGATTGACGGCCCTAT	+	68	59.61	44
	GGCACACGTGCCGCTTTACT	-		60.24	
Porphyromonas gingivalis	GCGAGAGCTGAACCAGCCA	+	90	59.07	44
	ACTCGTATCGCCGTTATCCCGTA	-		59.44	
Treponema denticola	TAAGGGACAGCTTGCTCACCCCTA	+	55	58.84	44
	CACCCACGCGTTACTACCAGTC	-		59.76	
Tannerella forsythia	CGATGATACGCGAGGAACCTTACCC	+	72	59.07	44
	CCGAAGGGAAGAAAGCTCTCACTCT	-		58.01	
Aggregatibacter actinomycetemcomitans	GTGGGGAGCAAACAGGATTAG	+	260	58.63	77
	CCTAAGGCACAAACCATCTC	-		58.63	

T_m, melting temperature

water) for 15 min, rinsed with distilled water, stained with solution B (1 g phosphomolybdic acid (Sigma-Aldrich, 79560) in 100 ml distilled water) for 5 min, rinsed with distilled water, stained with solution C (2 g light green SF yellowish (Sigma-Aldrich, 62110) and 2 ml glacial acetic acid in distilled water to a final volume of 100 ml) for 1 min, rinsed with distilled water, and dehydrated with 100% ethanol.

Scanning electron microscopy

For SEM analysis of the 3D culture, slices of harvested collagen sponges were introduced into 0.185 M Sodium cacodylate buffer for one day, dissected and left for 1 day in cacodylate before being immersed in 2.5% glutaraldehyde in cacodylate for 45 min, washed in cacodylate for 1 h, 5 min in H₂O, and then stored at 4°C overnight in 50% acetone. The samples were later dehydrated in a graded series of acetone, dried in CO₂, and thereafter mounted on aluminum stubs and sputter-coated with 4 nm of platinum. The samples were imaged using a TESCAN VEGA TS 5136 XM scanning electron microscope (Tescan) at 25 KV.

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Statistical evaluation

All data present in the experiment derives from triplicate biofilm cultures. For the bacterial determination by qPCR, the values were logarithmically transformed, and then inserted to Prism v.6 software (GraphPad, La Jolla California USA). The statistical differences ($P \leq 0.05$) between the groups were calculated by student t-test. For the determination of cytokine concentrations, the statistical differences ($P \leq 0.05$) between the groups were calculated by student t-test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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