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## Strain-Specific Colonization Patterns and Serum Modulation of Multi-Species Oral Biofilm Development

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### Abstract

Periodontitis results from an ecological shift in the composition of subgingival biofilms. Subgingival community maturation is modulated by inter-organismal interactions and the relationship of communities with the host. In an effort to better understand this process, we evaluated biofilm formation, with oral commensal species, by three strains of the subgingivally prevalent microorganism *Fusobacterium nucleatum* and four strains of the periodontopathogen *Porphyromonas gingivalis*. We also tested the effect of serum, which resembles gingival exudates, on subgingival biofilms. Biofilms were allowed to develop in flow cells using salivary medium. We found that although not all strains of *F. nucleatum* were able to grow in mono-species biofilms, forming a community with health-associated partners *Actinomyces oris* and *Veillonella parvula* promoted biofilm growth of all *F. nucleatum* strains. Strains of *P. gingivalis* also showed variable ability to form mono-species biofilms. *P. gingivalis* W50 and W83 did not form biofilms, while ATCC 33277 and 381 formed biofilm structures, but only strain ATCC 33277 grew over time. Unlike the enhanced growth of *F. nucleatum* with the two health-associated species, no strain of *P. gingivalis* grew in three-species communities with *A. oris* and *V. parvula*. However, addition of *F. nucleatum* facilitated growth of *P. gingivalis* ATCC 33277 with health-associated partners. Importantly, serum negatively affected the adhesion of *F. nucleatum*, while it favored biofilm growth by *P. gingivalis*. This work highlights strain specificity in subgingival biofilm formation. Environmental factors such as serum alter the colonization patterns of oral microorganisms and could impact subgingival biofilms by selectively promoting pathogenic species.

### Keywords

oral multi-species communities; subgingival; *Fusobacterium nucleatum*; *Porphyromonas gingivalis*; strain variability; serum; biofilms

### Introduction

Oral microorganisms form complex biofilm communities in the subgingival environment, using the root surface as substratum for attachment and saliva and gingival crevicular fluid

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(GCF) as nutritional sources. Microorganisms in these communities interact physically, via adhesin-receptor mediated attachment, and metabolically, via cross-feeding, collective degradation of host macromolecules and exchange of metabolic signals [1–6]. Since it is nearly impossible to reconstruct in vitro the great complexity of subgingival microbial communities, the development of simplified models is fundamental to start dissecting the interactions that lead to community maturation and dysbiosis associated with periodontal disease.

*Fusobacterium* spp. are an important component of subgingival communities comprising ~10 to ~20% of the total flora, regardless of periodontal health status [7 and unpublished data from our laboratory]. *Fusobacterium nucleatum* appears to be the most abundant fusobacteria in the subgingival environment [8]. Due to its time of appearance during undisturbed plaque accumulation, abundance in plaque, coaggregation profile and ability to rapidly reduce the oxidative potential of the environment, *F. nucleatum* has been proposed to be important in the transition from health-associated communities to those associated with disease [9–11]. Modeling such transitions and dissecting the role of *F. nucleatum* in community maturation are thus important tasks if we are to gain a better understanding of the ecology of subgingival biofilm communities.

Among the bacterial species associated with periodontitis, *Porphyromonas gingivalis* plays a prominent role in the onset and progression of the disease [12, 13]. *P. gingivalis* virulence has been attributed to a great variety of factors involved in processes such as nutrient acquisition, immune activation and host evasion [14]. Some attention has also been paid to the process by which this microorganism establishes itself within subgingival biofilm communities. It seems that a pre-established community of other bacteria has an important role facilitating retention of *P. gingivalis* in vivo [15]. Furthermore, using a flow cell system with saliva as sole nutritional source, Periasamy and Kolenbrander [16] demonstrated that partnering with another oral species was in most cases beneficial and allowed *P. gingivalis* ATCC 33277 to increase its biofilm biomass over an 18 hour period. *P. gingivalis* has also been shown to form biofilms with the initial colonizer *Streptococcus gordonii*; however, in response to the presence of the streptococci, *P. gingivalis* decreases transcription of adhesion and signaling genes, a possible indication of switching to a “detachment” phenotype [6, 17]. It is thus likely that other community members modulate the establishment of *P. gingivalis* in the subgingival environment.

A less studied aspect of oral biofilm development is the variability among different strains from the same species in their colonization patterns. For example, strains of *F. nucleatum* appear to have heterogeneous coaggregation patterns, an indication of intra-species variability in their ability to recognize cell surfaces from other genera [18]. Variable cell surface characteristics are also likely to affect biofilm formation. Moreover, the population structure of *P. gingivalis* is highly heterogeneous from a genotypic perspective [19, 20]. Phenotypically, heterogeneity in *P. gingivalis* virulence has been observed with some strains considered “virulent” due to their ability to disseminate systemically, as shown using the subcutaneous mouse abscess model [21]. According to this assay, the type strains *P. gingivalis* W83 and W50 are considered “virulent”, while *P. gingivalis* 381 is considered “avirulent”, as it only produces localized abscesses [21]. Interestingly, genotypically similar strains to those classified as “avirulent” using the subcutaneous abscess model are more prevalent in subgingival plaque of periodontally healthy subjects, while the “virulent” genotypes occur more frequently in subjects with periodontitis. [20]. These differences have been attributed to the presence in “virulent” strains of a capsular polysaccharide structure, which decreases bacterial attachment, biofilm formation and the effectiveness of host cell responses, thus facilitating systemic spread [22–24]. A question that remains unanswered, however, is how encapsulated “virulent” strains colonize and persist in the oral environment,

since an ability to form biofilms is a prime requirement for any bacterial species to maintain its population levels in the oral cavity, where saliva and GCF flow constantly.

Subgingival biofilm communities receive a constant influx of GCF, a serum-derived exudate that flows at a higher rate when local inflammation develops at the gingival margin [25]. Main components of GCF include albumin, transferrin, immunoglobulins and defensins, among many others [26, 27]. The role of serum, as an in vitro substitute for GCF, in the physiology of subgingival microorganisms has been studied in batch-wise enrichment cultures of subgingival plaque [28]. These studies demonstrated that several enrichment steps lead to the accumulation of bacteria classified, at the time, as *Bacteroides* spp., peptostreptococci and fusobacteria. Enriched natural consortia collectively degraded serum macromolecules, a pattern also observed with artificially assembled consortia [29]. These studies suggest that serum may promote the growth of certain subgingival species. However, the effect of serum on adhesion and biofilm formation of oral microorganisms is less clear. Human serum has been shown to inhibit the attachment of *P. gingivalis* to hydroxyapatite surfaces treated with type I collagen [30], however the effect of serum on adhesion and biofilm formation of subgingival consortia has not been evaluated.

Accordingly, the goal of this study was to characterize biofilm formation by different strains of *F. nucleatum* and *P. gingivalis*. We were also interested in testing whether the presence of other community members affected colonization patterns by these strains and in evaluating the role of serum on multi-species biofilms. In order to accomplish these goals we first tested three *F. nucleatum* strains for their ability to form mono-species or multi-species biofilms with health-associated partners *Actinomyces oris* and *Veillonella parvula*. Next, we evaluated the ability of four *P. gingivalis* strains to form mono-species biofilms and integrate into a 4-hour-old health-associated biofilm community in the presence and absence of *F. nucleatum*. We then evaluated the role of native serum as a potential environmental factor that modifies the characteristics of multi-species subgingival communities. These studies were aimed at increasing our understanding of the processes involved in subgingival biofilm development by the establishment of models representative of in vivo conditions, where transitions from a health-associated to a pathogenic biofilm could be investigated.

## Methods

### Microorganisms and microbiological media

The microorganisms used in this study were *Actinomyces oris* T14V, *Veillonella parvula* PK1910, *Fusobacterium nucleatum* subsp. *polymorphum* ATCC 10953, *Fusobacterium nucleatum* subsp. *polymorphum* 12230, *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586, *Porphyromonas gingivalis* W83, *Porphyromonas gingivalis* W50 (ATCC 53978), *Porphyromonas gingivalis* ATCC 33277 and *Porphyromonas gingivalis* 381. *A. oris* was grown in brain heart infusion (BHI) medium. *V. parvula* was grown in BHI medium supplemented with 0.17 M lactic acid, 0.5 g liter<sup>-1</sup> cysteine and 5 mg liter<sup>-1</sup> hemin. *F. nucleatum* strains were grown in BHI medium supplemented with 0.5 g liter<sup>-1</sup> cysteine. *P. gingivalis* strains were grown in tryptic soy broth (TSB) supplemented with 0.5 g liter<sup>-1</sup> cysteine, 5 mg liter<sup>-1</sup> hemin and 1 mg liter<sup>-1</sup> menadione. All microorganisms were grown at 37°C in an anaerobic chamber with an atmosphere of H<sub>2</sub>:CO<sub>2</sub>:N<sub>2</sub> (5:5:90).

Saliva, used to supplement the biofilm growth medium was collected from 10 systemically healthy volunteers according to a protocol approved by the Institutional Review Board of the University of Connecticut Health Center (IRB #10-216-2). Methods for saliva processing and storage have been previously described [31].

## Flow cell experiments

Biofilm growth of microorganisms was evaluated using flow cells harboring a 24 × 60 mm glass cover slip as the attachment substratum. General procedures for flow cell set up and sterilization have been described previously [31]. Briefly, prior to each experiment, flow cells were cleaned with 0.1 M HCl and rinsed with sterile distilled water. Subsequently, 10% (vol/vol) hypochlorite was pumped for 2 hours followed by a rinse with sterile distilled water overnight. The flow cells were then placed at 37°C in an anaerobic chamber and treated with saliva-supplemented growth medium (22.5% (vol/vol) sterile human saliva and 10% (vol/vol) BHI, supplemented with 0.05 g liter<sup>-1</sup> cysteine, 0.5 mg liter<sup>-1</sup> hemin and 0.1 mg liter<sup>-1</sup> menadione, in PBS) for 15 min to allow formation of a salivary pellicle on the glass surface. Saliva diluted to a similar concentration has been shown to support the growth of oral biofilms [16, 32, 33]. When the effect of serum was evaluated, 10% (vol/vol) commercially available human native serum (Mediatech, Inc., Manassas, VA) was added to saliva-supplemented growth medium. This serum concentration is reported to support biofilm formation by *P. gingivalis* [34]. For experiments with serum, unless otherwise indicated, medium containing serum was used for resuspension of the bacterial inoculum and at all times during the flow cell experiment. For some experiments, saliva-supplemented growth medium without serum was used for inoculum resuspension, during the conditioning period and attachment of microorganisms and serum was only introduced to the system in the flow, after initial attachment had occurred.

For inoculation of flow cells, bacterial cultures were allowed to grow until late logarithmic phase, which was defined as an OD<sub>600nm</sub> equal to 1.3 for *A. oris*, *V. parvula* and *F. nucleatum* and an OD<sub>600 nm</sub> equal to 1.1 for *P. gingivalis*. Bacterial cultures were then normalized to an OD<sub>600 nm</sub> equal to 1.0 and microbial cells were washed in salivary growth medium, a step that has been shown to increase attachment of cells to the conditioned surface [32]. Flow cells were inoculated with mono-, dual- or multi-species inocula. The inoculum for each flow cell was prepared by mixing appropriate combinations of 1 × 10<sup>7</sup> cells of each species. After injecting the inoculum, flow cells were inverted and microorganisms were allowed to attach for 30 minutes under static conditions. Flow cells were then placed upright and the pump started with a flow rate for all experiments of 100 μL min<sup>-1</sup>. Biofilms were allowed to develop for 4 and 16 hours. For multi-species biofilms containing *P. gingivalis*, the microorganism was inoculated after 4 hours of biofilm development by previously inoculated species, unless otherwise indicated. Biofilms were allowed to develop for an additional 4 and 16 hours after *P. gingivalis* inoculation. All flow cell experiments were repeated at least three times.

## Staining, imaging and quantification of biofilms

Biofilms were fixed in 4% paraformaldehyde at 4°C for 3 hours. Biofilms were then stained by fluorescence in-situ hybridization (FISH) with oligonucleotide probes labeled with Alexa dyes. Briefly, flow cells were washed with D-PBS and bacterial cells were permeabilized with lysozyme (70,000 U mL<sup>-1</sup> in 100 mM Tris/HCl pH 7.5 and 5 mM EDTA) for 10 min, at 37°C. Biofilms were then dehydrated in a series of ethanol washes and exposed to hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl pH 7.5, 0.01% sodium dodecyl sulfate and 25% formamide) containing 10 ng mL<sup>-1</sup> of probe. Biofilms were then incubated at 46°C for 90 min and washed for 15 min at 48°C in washing buffer (20 mM Tris/HCl pH 7.5, 5 mM EDTA, 0.01% sodium dodecyl sulfate and 159 mM NaCl). The oligonucleotide probes JF201 [35], VEI488 [36], FUS664 [35] and Bg-8 [37] were used to detect *A. oris*, *V. parvula*, *F. nucleatum* and *P. gingivalis*, respectively. Biofilms were visualized with a Zeiss LSM 510 confocal scanning laser microscope equipped with an argon laser, using a water immersion C-Apochromat 40× objective (NA1.2). Stacks of z-plane images from at least 8 different fields of view per sample were acquired and later reconstructed into 3D images

using IMARIS software (Bitplane Inc., Saint Paul, MN). Surface reconstructions using the surpass mode were used to calculate the biovolume (in  $\mu\text{m}^3$ ) of each microorganism. Significant growth from early to late time points was evaluated via student's t-test. Differences in biovolumes of species across conditions, at specific time points, were also evaluated via student's t-test.

### Planktonic growth curves

Planktonic growth of *F. nucleatum* ATCC 10953 in mono-culture or in the presence of a community of *A. oris* and *V. parvula* was evaluated by inoculating  $10^6$  cells of each microorganism into triplicate test tubes containing 10 mL of pre-reduced saliva-supplemented growth medium in which the vol/vol of BHI was 30%. Cultures were incubated at 37°C under anaerobic conditions until stationary phase was reached. *F. nucleatum* was quantified by sampling microbial cultures at various time points. Culture samples were sonicated for 10 s, as previously described [31], to disrupt microbial aggregates. Samples were then serially diluted in PBS and plated on BHI-blood agar. *F. nucleatum* was identified in mixed-culture samples by colony morphology.

In order to assess the effect of serum on viability, microorganisms were grown planktonically in the absence or presence of native human serum.  $10^6$  bacterial cells were inoculated into pre-reduced microorganism-specific growth medium supplemented with either 10% human serum or D-PBS. Triplicate cultures for each condition were incubated anaerobically at 37 °C until stationary phase was reached. Growth was monitored by determining  $\text{OD}_{(600\text{nm})}$ .

### Coaggregation assays

Coaggregation among strains used in this study was evaluated visually according to the method of Cisar *et al.* [38]. Planktonic coaggregation was evaluated in saliva and also in coaggregation buffer. Scores for the degree of coaggregation ranged from 0 to 4 and were assigned by the following criteria: 0-no visible aggregates in the cell suspension; 1-small uniform coaggregates in suspension; 2-definite coaggregates easily seen but suspension remained turbid without immediate settling of coaggregates; 3-large coaggregates which settled rapidly leaving some turbidity in the supernatant fluid; 4-clear supernatant fluid and large coaggregates which settled immediately. Autoaggregation was also tested following these procedures and taken into account when interpreting coaggregation data.

## Results

### Strain-specificity in biofilm formation by *F. nucleatum* and its interaction with early colonizers *A. oris* and *V. parvula*

We first tested the ability of each of three strains of *F. nucleatum* to grow in a mono-species biofilm using flow cells and saliva-supplemented medium. As shown in Figure 1A, early biofilms (4h) did not differ among the three strains evaluated. However, mono-species biofilms of *F. nucleatum* ATCC 25586 and strain 12230 increased in biomass from 4h to 16h, while *F. nucleatum* ATCC 10953 was not able to grow as a mono-species biofilm between these time points. We then tested the effect of a community formed by *A. oris* and *V. parvula* on the biofilm growth of the three *F. nucleatum* strains. We confirmed results previously reported [39], in that *A. oris* and *V. parvula* formed dual-species biofilms in which both partners grew (Figure 1B). Figure 1B also shows that each strain of *F. nucleatum* grew as part of this community benefiting from the interaction with other community members. *F. nucleatum* ATCC 10953, unable to grow in mono-species biofilms, demonstrated a more than 10-fold increase in biovolume from 4h to 16h in the presence of *A. oris* and *V. parvula*. Although able to grow as a mono-species, *F. nucleatum* ATCC

25586 also benefited demonstrating an increased biovolume at both 4h and 16h time points as part of a community in comparison to mono-species biofilms (mono-species vs multi-species 4h  $P=0.017$  and 16h  $P=0.007$ ). Moreover, *F. nucleatum* 12230 showed an increase in biovolume of ~100 fold at both time points as part of a biofilm community in comparison to mono-species biofilms (mono-species vs multi-species 4h  $P=0.004$  and 16h  $P<0.001$ ).

With respect to biofilm architecture of microbial communities, all strains of *F. nucleatum* formed three-species inter-digitated structures with *A. oris* and *V. parvula* (Figure 1B, yellow arrow). Another commonly observed biofilm architecture arrangement was central colonies of *A. oris* surrounded by *V. parvula* in dual-species biofilms or by *F. nucleatum* and *V. parvula* in three-species biofilms (Figure 1B, white arrows in 3-D image projections and 2-D slices of  $x-z$  planes). These results highlight the synergy among these three species and the robust ability to form biofilms displayed by the three strains of *F. nucleatum* tested when part of a multi-species community.

To investigate further the mechanisms mediating the interaction between *F. nucleatum* and an *A. oris*/*V. parvula* community, we then tested the ability of *V. parvula* and *A. oris* to stimulate *F. nucleatum* ATCC 10953 in planktonic batch co-culture in a saliva-based medium. Figure 2A shows planktonic growth curves of *F. nucleatum* ATCC 10953 in the presence and absence of *A. oris* and *V. parvula*. Planktonic growth of *F. nucleatum* ATCC 10953 as part of a community in mixed-culture was slightly stimulated compared to mono-cultures. *F. nucleatum* entered logarithmic phase and reached stationary phase earlier in mixed-culture, the latter presumably because of nutrient exhaustion. Microscopic observation of planktonic mixed cultures revealed that the three microorganisms formed planktonic coaggregates of various sizes (Figure 2B). These findings demonstrate that the growth stimulatory effect of *A. oris* and *V. parvula* on *F. nucleatum* can also be detected in batch mixed cultures; however, the effect is more prominent in biofilms where microorganisms exist in close physical proximity under a flowing nutrient supply.

### Strain-specificity in mono-species biofilm formation by *P. gingivalis* and the effect of other community members on *P. gingivalis* colonization traits

Our next goal was to evaluate inter-strain variability in biofilm formation by four strains of the periodontal pathogen *P. gingivalis*. Additionally, we aimed to model a transition from a health-associated biofilm to a disease-associated biofilm by testing how different strains of *P. gingivalis* integrated into a community of health-associated early colonizers (*A. oris*-*V. parvula*), evaluating whether this community affected the colonization patterns of different strains. Since *F. nucleatum* is considered to play a central role in microbial succession, we tested its effect on *P. gingivalis* by including it in the community.

We found great inter-strain variability in *P. gingivalis* mono-species biofilm formation and biofilm growth capacity. As Figure 3 depicts, *P. gingivalis* W50 and *P. gingivalis* W83 displayed a poor ability to form biofilms, while *P. gingivalis* ATCC 33277 and 381 were able to attach to the saliva-conditioned surfaces and form biofilms. However, *P. gingivalis* ATCC 33277 grew between time points, while *P. gingivalis* 381 did not increase its biovolume between 4h and 16h. Insets on Figure 3 also depict differences in micro-colony size of adhered organisms with strains W50 and W83 showing micro-colonies of no more than 3  $\mu\text{m}$  in diameter, while strains ATCC 33277 and 381 formed micro-colonies of various sizes, some reaching ~40  $\mu\text{m}$ .

We then evaluated the ability of these four strains of *P. gingivalis* to integrate into a 4h-old biofilm of *A. oris* and *V. parvula* or a 4h-old biofilm of *A. oris*, *V. parvula* and *F. nucleatum*. For these experiments we first tested *F. nucleatum* ATCC 10953 since we previously identified a metabolic interaction occurring between this strain and *P. gingivalis* W50 [9].

As Figure 4 shows, *P. gingivalis* W50 and W83 did not improve in their ability to form biofilms in the presence of *A. oris* and *V. parvula* or when *F. nucleatum* ATCC 10953 formed part of the biofilm community (similar results observed for *F. nucleatum* 12230 and ATCC 25586; data not shown). Microscopic examination of planktonic coaggregation between *F. nucleatum* strains and *P. gingivalis* W83 or *P. gingivalis* W50 in the presence of saliva revealed minimal interaction of the two species. Furthermore, no coaggregation was observed between these two strains of *P. gingivalis* and *A. oris* or *V. parvula*, as tested by the visual coaggregation assay of cells suspended in saliva or buffer (see Methods; data not shown). These weak or absent cell-to-cell interactions might explain the lack of coadhesion of *P. gingivalis* W50 and W83 into preformed early biofilms.

The ability of *P. gingivalis* ATCC 33277 and 381 to integrate into a biofilm of *A. oris* and *V. parvula* (Figure 5, biovolume at 4h) was similar to their ability to form an early biofilm as mono-species (Figure 3, biovolume at 4h). However, no statistically significant increase in biovolume of *P. gingivalis* ATCC 33277 was observed between time points when forming part of a community with *A. oris* and *V. parvula* (Figure 5), in contrast to its growth in monospecies biofilms (Figure 3). As Figure 5 also shows, the presence of *F. nucleatum* ATCC 10953 showed a small but statistically significant stimulation of the ability of *P. gingivalis* ATCC 33277 to form biofilms as part of a community. Similar results were observed when *F. nucleatum* ATCC 10953 was replaced by either *F. nucleatum* 12230 or by ATCC 25586 (data not shown). Figure 5 also shows that *P. gingivalis* 381 was not affected by the presence of *A. oris* and *V. parvula* or *A. oris*, *V. parvula* and *F. nucleatum* ATCC 10953 and although it integrated into the biofilm community, it did not exhibit growth between time points, consistent with results of mono-species biofilms (Figure 3). Taken together, these results show that strains of *P. gingivalis* display a markedly different ability to form biofilms. Interaction with a community only affected strain ATCC 33277, while the other three strains tested were not influenced in their biofilm forming patterns.

### Serum as an ecological determinant of adhesion and biofilm architecture

We then evaluated whether the model biofilms developed were affected by the presence of human native serum, a nutritional source and possibly a limiting factor for the growth of microorganisms in the subgingival environment. We chose to use native serum, as opposed to heat-inactivated serum, to more closely simulate in vivo conditions. For these experiments, we inoculated flow cells simultaneously with *A. oris*, *V. parvula*, *F. nucleatum* ATCC 10953 and *P. gingivalis* 381. Figure 6 shows the effect of serum on multi-species biofilms and also on mono-species biofilms of *F. nucleatum* ATCC 10953 and *P. gingivalis* 381. The addition of serum to multi-species communities, during flow cell conditioning, attachment period and in the flow, resulted in a marked decrease in the biovolumes of *F. nucleatum* at both time points evaluated compared to the non-serum condition (4h  $P=0.0002$ ; 16h  $P=0.005$ ; Figure 6, two left panels), although *F. nucleatum* was still able to grow between time points in the presence of serum. A similar result was observed for *F. nucleatum* mono-species biofilms with a dramatic decrease in biovolume in the presence of serum (Figure 6, middle panel) compared to mono-species biofilms formed in the absence of serum (Figure 1A). On the contrary, in multi-species biofilms, biovolumes of *A. oris*/*V. parvula* or *P. gingivalis* 381 were not affected by the presence of serum at 4h when compared to the control condition. However, between time point comparisons showed that in the presence of serum, *A. oris*/*V. parvula* decreased in their biofilm biomass from 4h to 16h, while we observed for the first time an increase in *P. gingivalis* 381 biovolume between time points. Figure 6 also shows that serum also allowed *P. gingivalis* 381 to grow as a mono-species biofilm, in sharp contrast to results obtained in the absence of serum (Figure 3). These results suggest that serum has a selective effect on members of microbial

communities favoring biofilm growth by some species such as *P. gingivalis* and limiting attachment and biofilm formation by others such as *F. nucleatum*.

We then investigated if the observed decrease in biovolumes of *F. nucleatum* in the presence of serum was the result of an effect on adhesion, as serum was present during conditioning of flow cells and attachment, or alternatively serum affected viability of *F. nucleatum*. To test the role of serum in inhibition of bacterial attachment, we performed an experiment in which we conditioned flow cells and inoculated microorganisms in the presence of serum-free salivary medium, thus allowing the formation of an acquired pellicle over the substratum devoid of serum components. We introduced serum in the flow only after the attachment period of microorganisms had elapsed. As Figure 6 shows, when serum was introduced only in the flow, *F. nucleatum* biovolumes were still significantly lower than in the absence of serum (no serum vs serum during flow 4h  $P=0.0004$ , 16h  $P=0.01$ ), but higher than when serum was present during adhesion (serum during adhesion and flow vs serum during flow 4h  $P=0.06$ ; 16h  $P=0.0005$ ). These results suggest that the effect of serum on *F. nucleatum* is mostly due to an inhibition of its adhesion to the initially-formed pellicle. However, to rule out an effect on viability, we grew microorganisms planktonically in the presence and absence of serum. As Figure 7 demonstrates, serum did not affect viability of *F. nucleatum*.

Figure 7 also shows that in planktonic culture serum did not affect the viability of *A. oris* and *V. parvula*. However, serum decreased the growth rate of *P. gingivalis* 381, without completely inhibiting its growth as it reached a final OD similar to the no-serum condition. The negative effect of serum on *P. gingivalis* in planktonic culture contrasts with its positive effect on *P. gingivalis* 381 biofilm growth over time, particularly when serum was part of the initial pellicle (Figure 6). However, when microorganisms were inoculated as a multi-species biofilm under serum-free conditions, and serum was introduced only in the flow, *P. gingivalis* 381 failed to grow. These results confirm that the main effect serum exerts on biofilm microorganisms is mediated by its integration into the acquired pellicle covering the surface where microorganisms attach and develop biofilms. Collectively, these results suggest human native serum is a strong ecological determinant of subgingival biofilm formation.

## Discussion

Microorganisms in the oral cavity survive by attaching to oral surfaces. This obligatory biofilm lifestyle differs from a planktonic existence in that microorganisms must be able to display surface characteristics that allow them to attach to the substratum or to neighboring cells. Moreover, in biofilms, microorganisms encounter competition for resources from other cells living in their vicinity and must adapt to the beneficial or deleterious effects of metabolites released from other community members. These factors may explain the non-random association patterns seen in vivo in undisturbed dental plaque, where a remarkably intricate architectural organization is observed with individual species occupying specific physical niches [1, 40, 41]. Spatial organization of oral communities has only been studied at the species level; however, it is likely that because of intra-species population heterogeneity, strain variability exists in biofilm formation and inter-species association patterns. Here, we use an in vitro model of oral biofilm communities to study colonization patterns displayed by different strains of *F. nucleatum* and *P. gingivalis*, either as mono-species or as part of a community. We show that both *F. nucleatum* and *P. gingivalis* display intra-species variability in biofilm formation. A genome sequence comparison of three *F. nucleatum* subspecies has shown that the population of *F. nucleatum* is highly variable with the three subspecies compared only sharing ~62% of genes [42]. It is thus difficult to point to specific candidate genes responsible for the variable phenotype observed. Moreover, in



our study, strains ATCC 10953 and 12330 showed a different biofilm phenotype, although they both belong to the *polymorphum* subspecies. On the contrary, the genomes of the “virulent” strain *P. gingivalis* W83 and that of the “avirulent” strain *P. gingivalis* ATCC 33277 only differ by 7% [43]. Among the variable genes are those encoding for enzymes involved in capsular polysaccharide biosynthesis, which Davey and Duncan [23] later showed to be responsible for the “poor biofilm former” phenotype of *P. gingivalis* W83. It is thus likely that the inability to form biofilms of *P. gingivalis* W50, a strain genotypically very similar to W83 [20] is also due to the presence of a capsule polysaccharide.

The inability of *F. nucleatum* ATCC 10953 to grow as a mono-species biofilm in saliva-supplemented flow cells has been documented before [33]. The Kolenbrander laboratory also reported that a lack of biofilm growth by *F. nucleatum* ATCC 10953 could be relieved by the independent presence of either *A. oris* [33] or *V. parvula* [44], both of which are abundant components of subgingival plaque, particularly during periodontal health [7, 8, 45]. Our study shows that despite variability among *F. nucleatum* strains in their biofilm forming ability as mono-species, the presence of community members *A. oris* and *V. parvula* was beneficial, not only relieving the inability of *F. nucleatum* ATCC 10953 to grow in biofilms, but also facilitating biofilm formation by the two other strains tested, a suggestion of a potentially species-wide synergistic interaction. This synergistic community correlates with the in vivo abundance of these three genera in subgingival plaque during health [7]. Moreover, the positive interaction observed between *A. oris*, *V. parvula* and *F. nucleatum* coincided with a display of specific physical association patterns among strains in these three-species communities, in which *A. oris* formed central chimney-like micro-colonies surrounded by *V. parvula* and *F. nucleatum*. This architectural arrangement suggests *A. oris* occupies a central role in community dynamics. Interestingly, in vivo observations have revealed similar architecture in 48h-old biofilms developing on glass slabs carried in the mouth of volunteers, in which *Actinomyces* sp. were observed to form columnar micro-colonies surrounded by streptococci and other unidentified bacteria [46]. A defined spatial structure has been experimentally shown to be important in maintaining the stability of microbial communities, as it allows for the creation of chemical gradients where maximum productivity of specific strains within a syntropic context is achieved [47]. In this respect, it was also interesting to observe that the synergistic effect of *A. oris* and *V. parvula* on *F. nucleatum* was more pronounced in multi-species biofilm communities than in planktonic batch cultures. Furthermore, it is also possible that a close physical association with *A. oris* and *V. parvula* increases the biofilm forming ability of *F. nucleatum* and thus, its increased biomass in a flow cell community is not only a consequence of an augmented growth capacity due to effective resource utilization but rather a change in gene expression of adhesion-related genes. The nature of this synergistic interaction is currently under investigation.

Different strains of *P. gingivalis* also displayed marked variability in their biofilm forming capacity. These differences are in agreement with previous observations of *P. gingivalis* mono-species biofilms formed on plastic surfaces in a closed environment [23]. The presence of a community did not markedly benefit the “poor biofilm former” strains of *P. gingivalis* W83 and W50, which were almost undetectable in biofilms under all conditions tested. Although *P. gingivalis* can fulfill its role as a pathogenic species even at low levels of oral colonization [48], persistence in the oral cavity would still require a minimal capacity to form biofilms on oral surfaces. Thus, the mechanisms by which *P. gingivalis* “virulent” strains colonize the tooth surfaces remain unclear. It is possible that biofilm formation by these strains requires synergy with specific partners or alternatively, environmental conditions different to the ones tested in this study modulate in vivo colonization by *P. gingivalis* encapsulated strains.

Contrary to the lack of multi-species biofilm formation by *P. gingivalis* W50 and W83, the “avirulent” but “good biofilm-formers” *P. gingivalis* ATCC 33277 and 381 were able to integrate into a community of early health-associated colonizers, achieving similar biovolumes at the 4h time point as those achieved in early mono-species biofilms. However, the presence of *A. oris*/*V. parvula* impeded biofilm growth by *P. gingivalis* ATCC 33277, unless *F. nucleatum* was part of the community. On the contrary, the presence of the middle colonizer *F. nucleatum* did not markedly change the biofilm growth of *P. gingivalis* 381. *F. nucleatum* has been proposed to have a fundamental role in the establishment of periodontal pathogens during microbial succession events, based on in vivo and in vitro observations [9, 12, 49, 50]. The lack of observation in this study of a species-wide effect of *F. nucleatum* on the growth of *P. gingivalis*, highlights the specificity of inter-organismal interactions. Moreover, it is possible that different environmental conditions to those used in this study could enhance the ability of *F. nucleatum* to metabolically support *P. gingivalis*. For example, the positive interaction we previously reported in planktonic continuous cultures between these two species, where *F. nucleatum* supplied CO<sub>2</sub> to *P. gingivalis* and reduced the environment, allowing the latter to grow under more oxygenated conditions than those it tolerates as a mono-culture [9], could not have occurred under the CO<sub>2</sub>-rich anaerobic conditions used in the present study. Furthermore, the previously reported ability of *F. nucleatum* to allow the biofilm growth of *P. gingivalis* ATCC 33277 in flow cells was tested in a system with saliva as the sole nutrient supply [16], in contrast with the current experiments in which the salivary medium was supplemented with BHI, hemin and cysteine, a condition that supported mono-species biofilm growth by *P. gingivalis* ATCC 33277. It is also possible that the health-associated community of *A. oris* and *V. parvula* changed *P. gingivalis* phenotype towards one compatible with detachment, as occurs when *P. gingivalis* forms biofilms over a surface previously colonized by *S. gordonii* [6]. A situation consistent with a switch towards detachment is possibly indicated by the decreased micro-colony size displayed by *P. gingivalis* ATCC 33277 and 381 when forming part of a community in comparison to mono-species biofilms (Figures 4 and 6).

In this study we also show that the presence of serum alters colonization patterns by oral microorganisms. Bovine serum and bovine serum albumin have been shown to inhibit the adhesion to abiotic surfaces and subsequent biofilm development of non-oral species *Pseudomonas aeruginosa* [51] and *Helicobacter pylori* [52]. Similarly, in this study, we found that when human serum was part of the acquired pellicle formed over the glass substratum, it had a marked negative effect on the adhesion of *F. nucleatum*, while it did not affect the initial adhesion of *P. gingivalis* or *A. oris*/*V. parvula*. This is thus an indication that the main role of the gingival crevicular exudate flowing from the adjacent gingival tissues and constantly bathing the tooth surfaces is to serve as an innate immunity defense mechanism limiting adhesion of certain species. Interestingly, serum as part of the substratum allowed *P. gingivalis* 381 to form a commensal interaction with the three-species community, growing as part of the community and displaying micro-colonies of greater size, similar to those observed in mono-species biofilms. Importantly, the presence of serum only in the flowed medium did not replicate this result, an indication that the surface characteristics of the initially formed pellicle determine the type of biofilm that develops on the surface. This may explain why eradication of periodontal pathogens from tooth surfaces is a challenge, as after removal of these biofilms via dental procedures such as scaling and root planning, the bleeding tissues and inflammatory fluids immediately form a pellicle over the debrided tooth surface that favors the reestablishment of a pathogenic biofilm. It is thus important that future studies address the role of serum on biofilm formation by other periodontal pathogens.

In conclusion, these studies demonstrate that strain specificity in biofilm formation exists and should be considered when establishing models of subgingival plaque. Importantly, this

variability in biofilm formation can be modulated by species-specific interactions with other community members, as demonstrated by the synergistic effect of *A. oris* and *V. parvula* on *F. nucleatum* that allowed all strains to grow as a biofilm. In contrast, *P. gingivalis* variability in biofilm formation remained when part of a multi-species community. Our experiments also suggest that environmental factors, such as the presence of a serum-like gingival exudate could have profound effects on microbial adhesion to surfaces and in the ability of certain microorganisms to flourish in the biofilm community.

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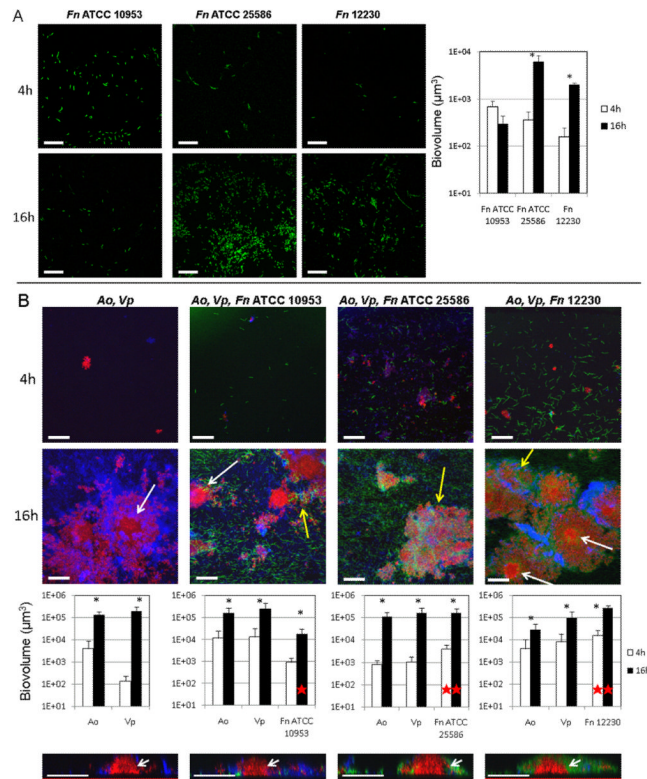
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**Highlights**

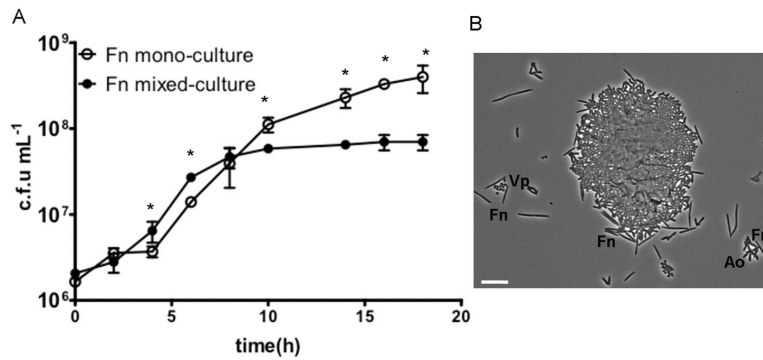
- We studied the role of strain variability and serum on oral biofilm formation.
- Strains of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* differ in their ability to form monospecies biofilms.
- *Actinomyces oris* and *Veillonella parvula* allow all strains of *F. nucleatum* tested to grow as biofilms.
- Growth as part of a community does not markedly change inter-strain variability in *P. gingivalis* biofilm formation.
- Serum limits biofilm formation by *F. nucleatum*, while it enhances *P. gingivalis* biofilm growth.



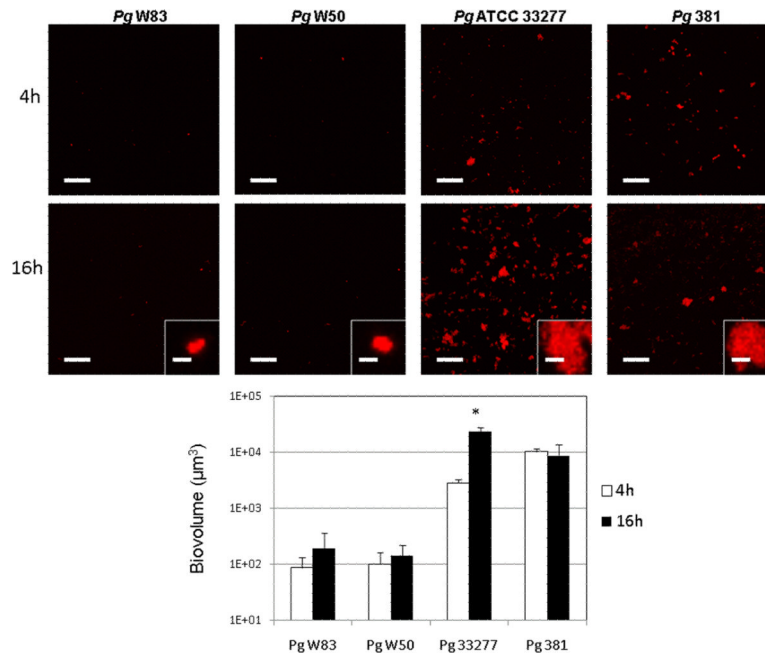
**Figure 1.**

Mono-species and multi-species biofilm formation by three strains of *F. nucleatum*. Biofilms were allowed to develop for 4 and 16h in flow cells using saliva-supplemented medium as nutritional source. Panel A depicts representative CLSM 3-D image projections and biovolume measurements (in  $\mu\text{m}^3$ ) for mono-species biofilms formed by three strains of *F. nucleatum* (green). Panel B depicts multi-species biofilm formation by *F. nucleatum* strains (green) with initial colonizers *A. oris* (red) and *V. parvula* (blue). White arrows in 16h CLSM 3-D image projections and 2-D *x-z* planes slices (bottom) indicate central columnar micro-colonies of *A. oris*. Yellow arrows indicate three-species inter-digitated structures. *F. nucleatum* = *Fn*, *A. oris* = *Ao* and *V. parvula* = *Vp*. \* indicates a *P* value of less than 0.05 when biovolumes for each species were compared between 4h and 16h. A red star indicates a *P* value of less than 0.05 when biovolumes of *F. nucleatum* were compared in mono-species biofilms vs. multi-species biofilms. Bar in all images = 50  $\mu\text{m}$ .

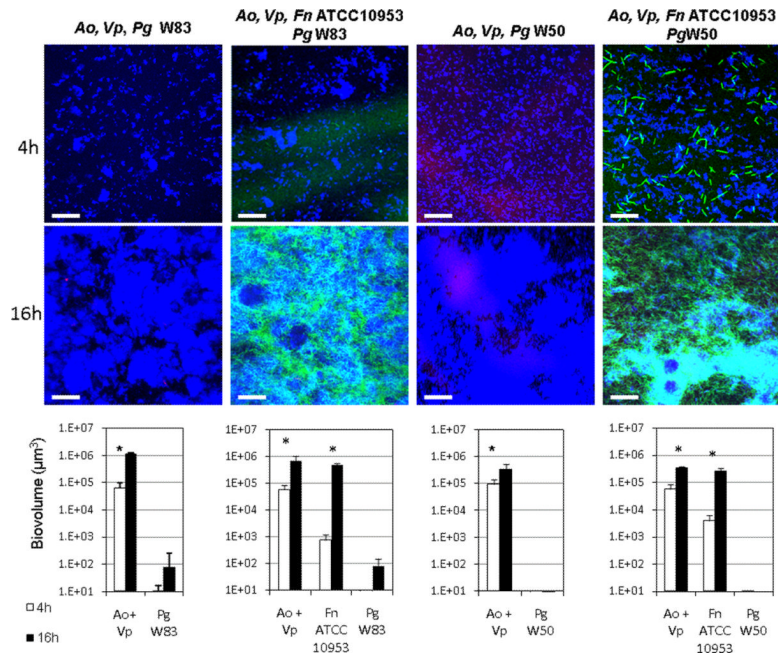




**Figure 2.** Planktonic growth of *F. nucleatum* ATCC 10953 in salivary growth medium as a mono-culture or in co-culture with *A. oris* and *V. parvula*. Panel A shows planktonic growth curves. Data points represent measurements from three independent replicate experiments. Panel B shows a representative phase contrast micrograph of a mixed-species culture after 10 hours of growth in saliva-supplemented medium. Short rods are *A. oris* (Ao); coccoid cells are *V. parvula* (Vp) and thin long rods are *F. nucleatum* (Fn). Scale bar = 10  $\mu\text{m}$ . \* indicates a *P* value of less than 0.05 when colony forming units (c.f.u)  $\text{mL}^{-1}$  of Fn were compared between mono-culture and co-culture conditions at each time point.

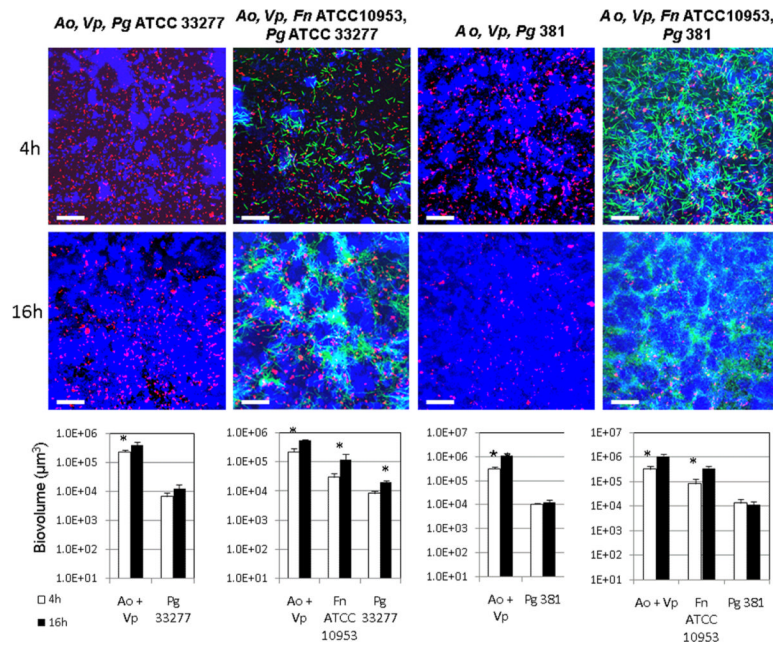


**Figure 3.** Mono-species biofilm formation by four strains of *P. gingivalis*. Biofilms were allowed to develop for 4 and 16h in flow cells using saliva-supplemented medium as nutritional source. *P. gingivalis* (*Pg*) appears in red in CLSM 3-D image projections. \* indicates a *P* value of less than 0.05 when biovolumes were compared between 4h and 16h. Left scale bar in all images = 50 μm. Scale bar in inset digital zoom images = 3 μm.



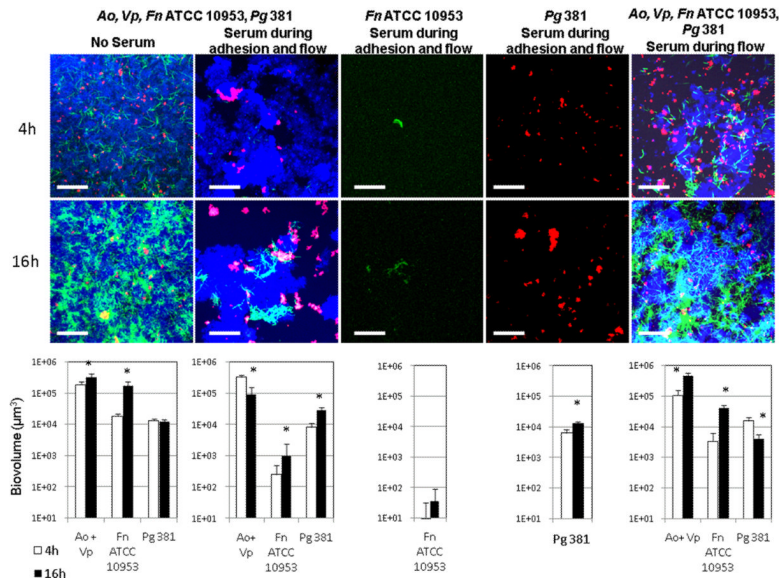
**Figure 4.**

Ability of virulent *P. gingivalis* strains W50 and W83 to integrate into 4h-old biofilm communities of health-associated species. In panel A, biofilms of *A. oris* and *V. parvula* or *A. oris*, *V. parvula* and *F. nucleatum* were allowed to develop for 4h in flow cells using saliva-supplemented medium as nutritional source. After 4h, *P. gingivalis* was inoculated, and biofilms were allowed to develop for an additional 4h and 16h. In CLSM 3-D image projections *A. oris* (*Ao*) and *V. parvula* (*Vp*) appear in blue, *F. nucleatum* (*Fn*) appears in green and *P. gingivalis* (*Pg*) in red. *A. oris* and *V. parvula* were quantified as a combined biovolume (*Ao* + *Vp*). \* indicates a  $P$  value of less than 0.05 when biovolumes were compared between 4h and 16h. Bar in all images = 50  $\mu\text{m}$ .

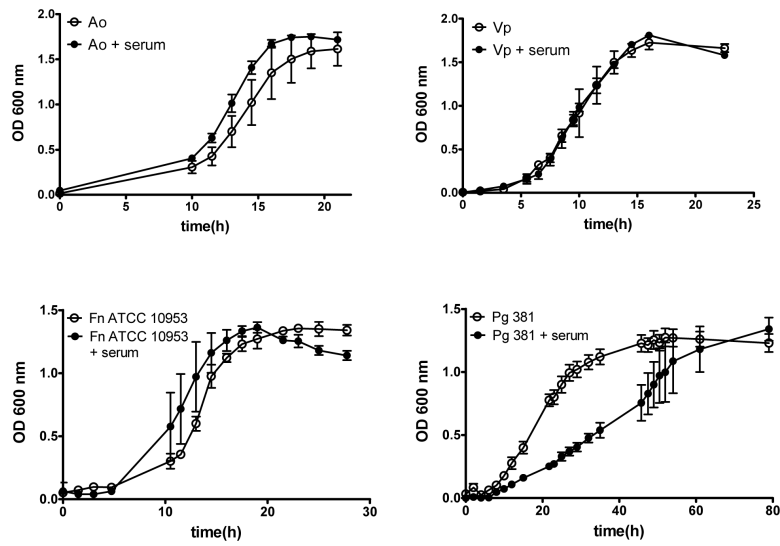


**Figure 5.**

Ability of avirulent *P. gingivalis* strains ATCC 33277 and 381 to integrate into 4h-old biofilm communities of health-associated species. Biofilms of *A. oris* and *V. parvula* or *A. oris*, *V. parvula* and *F. nucleatum* were allowed to develop for 4h in flow cells using saliva-supplemented medium as nutritional source. After 4h, *P. gingivalis* was inoculated, and biofilms were allowed to develop for an additional 4h and 16h. In CLSM 3-D image projections *A. oris* (*Ao*) and *V. parvula* (*Vp*) appear in blue, *F. nucleatum* (*Fn*) appears in green and *P. gingivalis* (*Pg*) in red. *A. oris* and *V. parvula* were quantified as a combined biovolume (*Ao* + *Vp*). \* indicates a  $P$  value of less than 0.05 when biovolumes were compared between 4h and 16h. Bar in all images = 50  $\mu\text{m}$ .



**Figure 6.** Effect of serum on mono-species and multi-species biofilm communities. Left image and biovolume data represent the control condition in which no serum was added to the salivary growth medium. In the three central images and graphs serum was present in the salivary medium during inoculum resuspension, substratum conditioning, attachment and in the medium flow. In right image and biovolume data graph, serum was only added to the salivary medium during flow. \* indicates a  $P$  value of less than 0.05 when biovolumes were compared between 4h and 16h. Bar in all images =  $65 \mu\text{m}$ . *A. oris* (*Ao*) and *V. parvula* (*Vp*) appear in blue, *F. nucleatum* ATCC 10953 (*Fn*) appears in green and *P. gingivalis* 381 (*Pg*) in red.



**Figure 7.** Effect of serum on viability of *A. oris* (Ao), *V. parvula* (Vp), *F. nucleatum* ATCC 10953 (Fn) and *P. gingivalis* 381 (Pg). Planktonic growth of microorganisms was evaluated in their appropriate nutrient-rich medium supplemented with 10% native human serum or with PBS as control. Data represent measurements from three independent replicate experiments.