# No man's land: Species-specific formation of "exclusion zones" bordering Actinomomyces graevenitzii microcolonies in nanoliter cultures

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**Abstract**

To survive within complex environmental niches, including the human host, bacteria have evolved intricate inter-species communities driven by competition for limited nutrients, cooperation via complementary metabolic proficiencies, and establishment of homeostatic relationships with the host immune system. Such complex, interdependent relationships have hampered attempts to culture many bacterial strains in research settings, where standard readout of co-culture experiments are usually limited to the relative abundance of each species. Here, we utilize a microfluidic-based co-culture system to characterize dynamic interactions between multiple oral bacterial isolates. Using time-lapse imaging, we define species-specific effects on spatial community relationships during co-culture of Streptococcus species and Staphylococcus aureus with Actinomyces species. Co-culture of Streptococcus cristatus or S. salivarius in nanoliter compartments with Actinomyces graevenitzii revealed localized exclusion of Streptococcus and Staphylococcus from media immediately surrounding A. graevenitzii micro colonies. This community structure did not occur with S. mitis or S. oralis strains, or in co-cultures containing other Actinomycetaceae species such as S. odontolyticus or A. naeslundii. Moreover, fewer neutrophils were attracted to compartments containing both A. graevenitzii and Staphylococcus aureus than to equal number of either species alone, suggesting a possible survival benefit from the interaction.

## Introduction

The complexity of bacterial communities that make up our microbiome mirrors the complexity of niches within human body. Of these niches, the oral cavity is perhaps one of the most diverse, presenting extremes of tissue stiffness, surface topography, transient temperature shifts, and nutrient flux[1]. Although the accessibility of the oral cavity has made it a focus of research into microbial community structure and diversity, our understanding of interspecies relationships and their role in health and disease remains limited.

A wide range of co-culture strategies have been developed to facilitate characterization of interspecies relationships, largely focusing on metabolic compatibility and coaggregation of species that form oral biofilms and plaque[2-4]. Efforts to culture previously "unculturable" species have focused on identifying co-culture partners that provide complementory metabolic functions to compensate for lack of specific metabolic pathways[5]. Physical distances and culture volume play key roles in metabolic symbiosis, interspecies communication, and cell-cell adherence[6]. Thus, multiple recent studies have used microfluidic approaches to achieve small-volume co-culture and to engineer co-culture devices with defined physical constraints[7-9].

Two bacterial genera commonly associated with oral biofilm formation are*Actinomyces* [10] and *Streptococcus* [11]. The genus*Actinomyces* has recently been subdivided with the creation of the genus Schaalia [12] with both *Actinomyces* and *Schaalia*being members of the family *Actinomycetacea* e. The bacteria*A. odontolyticus*

and Streptococcal spp. are considered early colonizers, adhering directly to the salivary pellicle coating the tooth surface. This facilitates the secondary adherence of intermediate colonizers, such as *Actinomyces* spp., followed by late colonizers such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis* during formation of dental plaque[6]. Sequential adherence of different bacterial species depends on their co-adhesion compatibility, which is often species-specific[13], while co-aggregation of bacterial species in suspension has been shown to directly influence gene expression to induce metabolic outputs to the benefit of both species[14]. Some bacterial species are incompatible for co-culture, leading to domination by one species at the expense of the other, often in a nutrient-dependent manner[15, 16].

Here, we utilized a previously-developed microfluidic device[17] to perform low-volume co-culture of multiple actinomyces and streptococcal species. Detailed microscopy revealed formation of defined "exclusion zones" surrounding *A. graevenitzii* microcolonies when co-cultured with *S. cristatus* or *S. salivarius* but not*S. oralis* or *S. mitis* . Additionally, exclusion zones were not observed with *S. odontolytica* or *A. naeslundii*in co-culture with any Streptococcal species tested, suggesting that the phenomenon exhibits a degree of species-specificity.

Interestingly, formation of exclusion zones around *A graevenitzii*microcolonies was also observed in co-culture with of*Staphylococcus aureus. S. aureus* is a common commensal present on the skin and upper respiratory tract of up to 50% of healthy individuals[18, 19]. It is also considered an important and dangerous opportunistic pathogen, due to high infection rates and the emergence of many antibiotic-resistant strains[20].

Innate immune cells, especially neutrophils, are the first cellular line of defense against *S. aureus* infection once physical barriers are breached[21]. As such, *S. aureus* infections induce a robust inflammatory response, which can lead to conditions such as cellulitis in the skin[22], more severe arthritic conditions following infections of the bones and joints[23], and sepsis. Studies have identified *S. aureus* in between 17-48% of healthy oral samples, with even higher rates of up to 64% present in young children[24]. Despite this, *S. aureus* is not considered a significant oral pathogen, and infections in the oral cavity are usually limited to inflammatory conditions such as angular cheilitis[25], along with rarer cases of jaw cysts[26] and oral mucosal lesions[27]. Systemic dissemination of *S. aureus* originating from the oral cavity remains a relatively unexplored topic.

Here, we characterized the interactions of several species of Actinomyces and Streptococcus in nanoliter confinement and observed the formation of exclusion zones between colonies, which were not observable in traditional co-cultures. Moreover, using a GFP-expressing strain of*S. aureus* , we observed that the innate immune responses to*S. aureus -A. graevenitzii* co-cultures were significantly dampened compared to *S. aureus* mono-cultures.

## Materials and methods

*Bacterial cell culture*

*Actinomyces graevenitzii* was cultured on Chacollate II agar (GC II Agar with hemoglobin [10 g/L] and IsoVltalex$^{TM}$[1% v/v]) (BD, USA) at 37°C in an anaerobic incubator. Single colonies from agar plates were picked and separately suspended in 10 mL of brain heart infusion (BHI) broth medium. *Streptococcus cristatus* was incubated at 37°C in the incubator with shaking overnight. Bacterial suspension concentrations were determined using a hemocytometer and the final concentration of bacteria was adjusted to $1 \times 10^7$ cells/mL by dilution in with Iscove's Modified Dulbecco's Medium IMDM supplemented with 20% Fetal Bovine Serum (FBS).

*Staphylococccus aureus* strain SH1000-GFP, which constitutively expresses green fluorescent protein (GFP), was received as a generous gift from the laboratory of Mary Mullins at the University of Sheffield (Sheffield, UK). Bacterial cultures were routinely cultivated in BHI Agar Plates with 5 µg/ml Tetracycline (Teknova, CA). Single colonies from agar plates were picked and suspended in 10 mL of BHI broth medium (Remel, Lenexa, KS, USA) with 5 µg/ml Tetracycline and then incubated at 37°C in aerobic incubator with shaking overnight. After overnight incubation, bacterial suspensions were sub-cultured by adding 1 mL of the

overnight culture into 49 mL of BHI broth with Tetracycline for 4 hours. Bacterial concentrations were determined using a hemocytometer and the final concentration of bacteria was adjusted to $5 \times 10^6$ cells/mL and diluted with IMDM supplemented with 20% FBS.

*Fabrication of microfluidic devices* :

Devices were fabricated using standard soft -lithography techniques on four-inch wafers. Photoresist (SU-8, Michrochem, Newton, MA) was spin-coated onto a silicon wafer and exposed to ultraviolet (UV) light, through a photolithography mask. Briefly, two layers of negative photoresist, the first 3 µm thin, the second 50 µm thick were patterned on a silicon wafer by sequentially employing two photolithography masks and processing cycles according to the instructions from the manufacturer. The silicon master wafer with photo patterned structures was employed to mold microchamber that were 200 µm in diameter, 50 µm in depth (Fig. 1A,B). To test effect of different depth another silicon master wafer with photo patterned structures was used to mold microchamber that were 200 µm in diameter, 10, 30,50 and 100 µm in depth. The entrance for each microchamber was 100 µm in length, 10 µm in width and 5 µm in depth. Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) was mixed with cross-linking agent in a ratio of 10:1 and poured onto wafers. The PDMS was cured overnight at 65°C, after which the PDMS layer was peeled off the wafer and the arrays of wells were cut using a scalpel and inlet, outlet was punched using 0.75 µm puncher. The microfluidic devices were bonded to glass-bottom 6-well plates after treating the bonding surface of PDMS and plate with oxygen plasma. The plates were heated to 70°C for 15 minutes to complete the PDMS-to-glass bonding. Each device consists of 99 chambers, uniformly distributed inside groups of three channels.

*Device loading*

Each PDMS device contained three sample chambers. When bonded to a 6-well glass-bottom plate, this allowed 18 conditions to be tested in parallel. Devices were first placed into a vacuum chamber for 20 mins prior to loading. To load the inner chambers, bacterial cocultures were then loaded into each main channel using a micropipette, and the devices allowed to equlibrate. We then checked that the bacteria had been successfully drawn into the inner chembers. If air bubbles still remained, the plate was placed under vacuum for further 5 minutes. During loading steps, care was taken to avoid mixing of different samples loaded into parallel chambers on the same PDMS device. After loading, the channels were washed thoroughly the media (IMDM with 20% FBS).

*Neutrophil isolation and neutrophil-microbe interactions*

Neutrophils were isolated from healthy donor blood (Research Blood Components, LLC, Watertown, MA) using a negative selection kit (StemCell Technologies, Inc. Cambridge, MA) according to manufacturers instructions. Cells were stained with Hoeschst 33342 (Thermo Fisher), washed, and resuspended at 40 x 10ˆ6 cells per mL for loading into the device. Devices were loaded with mono- or co-cultures of *A. graevenitzii* and *S. aureus* as described above. Following thorough washing of the main channel, neutrophils were then loaded and imaging commenced.

*Image processing, data acquisition, quantification, and analysis*

During the experiments, a glass-bottom 6-well plate (Micro Device Instruments, Avon, MA, USA) with microfluidic device was placed on a fully automated Nikon TiE microscope. The microscope was fitted with an incubator humidified and heated at 37°C. Images were acquired through 10x or 20x objectives in phase contrast. Growth of bacteria and bacteria movement were recorded using time-lapse imaging. Individual frames were recorded at an interval of 10 minutes at 10x, 20x, or 40x objectives for 24 hours. For detailed observations, images were also acquired every 10 or 30 seconds, using an oil-immersion 100x objective, for a minimum of 90 minutes. The experiments for this study were repeated up to ten times, including all control experiments. Time lapse image sequences were analyzed by FIJI (Fiji Is Just ImageJ, NIH). Results were plotted using Graphpad Prism V8.2.1 and Sigma Plot version 12. Error bars represent mean ± SEM.

**Results**

**Spatially configurable bacterial co-culture using a microfluidic device.**

Co-culture in nanoliter volumes enhances competition for nutrients, metabolite cross-talk between complementary species, and cross-suppression via antimicrobial and quorum-sensing molecules. To perform nanoliter co-cultures, we utilized microfluidic devices that consist of an array of 1.57 nL-volume cylindrical chambers (200 μm diameter x 50 μm height) connected to a single 50 μm high outer channel by a 125 μm long channel with a 10 x 10 μm cross-section (Fig. 1A, adapted from[17]). The co-culture chambers are primed with a bacterial suspension by applying vacuum to de-gas the PMDS and then flowing the suspension though the channels and into the chambers. Once the chambers are loaded, the outer channel is washed with fresh media. For co-culture experiments, species can be cultured together directly in the inner chamber (Fig. 1B). PDMS devices are optically transparent and are bonded directly to glass coverslips, allowing detailed imaging of interactions between species on the glass surface using an inverted microscope.

We cultured a GFP-expressing strain of *Staphylococcus aureus*inside the nanoliter chambers, and observed that it achieved confluence after approximately 6 hours at 37°C when loaded at a concentration of $1x10^6$ cells/mL[17]. To test whether the volume of the inner chamber effected bacterial growth, we fabricated a series of devices with altered chamber heights. The height of the outer chamber was adjusted to 200 μm to improved loading and washing steps, and the height of the inner chamber was tested at 10, 30, 50, and 100 μm, corresponding to 1.6, 9.42, 15.7, and 31.4 pL respectively. In these devices, *S. aureus* exhibited increasingly restricted, clustered growth patterns as the volume was reduced, suggesting rapid consumption of available nutrients or accelerated sensing of quorum signaling molecules in reduced volumes (Fig. S1A-E). The device with 50 μm high chambers and outer channels was utilized for all subsequent experiments unless otherwise stated.

**Exclusion zones form around *Actinomyces graevenitzii* microcolonies in co-culture with Streptococcus.**

Species from the family *Actinomycetaceae* and genus*Streptococcus* are amongst the most common isolated from oral biofilms, particularly dental plaque. Both *Actinomycetaceae* and*Streptococcal* species grew well as mono-cultures within our microfluidic devices. To study the co-culture characteristics of*Actinomycetaceae* and Streptococcal species in our microfluidic chambers, we co-loaded 3 species of *Actinomycetaceae* (3 strains of *S. odontolyticus* , 3 strains of *A. naeslundii* , and 2 strains of *A. graevenitzii* ) in combination with 4 species of*Streptococcus* (1 strain of *S. salivarius* , 3 strains of*S. mitis* , 2 strains of *S. oralis* , and 1 strain of *S. cristatus* ), 56 combinations in total (Fig. 2, Fig S2). Detailed microscopy of the microfluidic chambers was performed at 8 hours.

Observations in nanoliter chambers with co-cultures of either *S. cristatus* or *S. salivarius A64PA33* with *A. graevenitzii* (either *FO530* or *FO582* strains) revealed a striking absence of physical association between the species. Otherwise confluent streptococcal cells appeared to be unable to grow in proximity to *A. graevenitzii* microcolonies, resulting in formation of an "exclusion zone" bordering the *A. graevenitzii* (Fig 2C).Exclusion zones did not form around *A. graevenitzi* i microcolonies in co-culture with *S. mitis* (strains *ATCC 903* or*ATCC 49456* ) or *S. oralis* (strains *FFB47* or*FCB39* ), or around either of the other actinomyces or schaalia species tested (Fig 2B). These observations rule out any physical exclusion of streptococcus cells from space inhabited by actinomyces cells. Instead, the exclusion appears more likely due to local production of a toxic metabolite or inhibitory compound with considerable species specificity. Importantly, we did not observe any separation between macroscopic co-cultures performed using traditional co-culture protocols (Fig. S3).

**Exclusion zones form in co-cultures of *A. graevenitzii*and *S. aureus*.**

Formation of exclusion zones appeared to exhibit species specificity within the *Streptococcus* genus. To test whether this phenomenon might also occur for different major Firmicutes genera we co-cultured*A. graevenitzii* with the GFP-expressing strain of*Staphylococcus aureus* (*SH1000-GFP* ) that had previously been shown to grow in our device. We observed formation of exclusion zones around *A. graevenitzii* colonies in co-culture with *S. aureus* (Fig. 3A,B), demonstrating that this effect is not specific to streptococcal

species. Exclusion zones did not form in co-cultures of*S. aureus* and *A. naeslundii* , suggesting that this phenomenon is not common to all actinomyces (Fig. 3C). Importantly, the ability to use a GFP-labelled strain in our studies provided considerable benefits with respect to automated image analysis.

**Exclusion zones are formed by stressed *A. graevenitzii*microcolonies in nutrient competition with *S. aureus***

Exclusion zones could be easily visualized in co-cultures of *A. graevenitzii* and GFP-expressing *S. aureus* (Fig. 3B), allowing us to measure multiple aspects of the co-culture that we thought might influence exclusion zone formation.

Given that exclusion zones formed around each individual *A. graevenitzii* microcolony, we hypothesized that co-cultures containing increasing numbers of *A. graevenitzii* microcolonies would have decreasing amounts of *S. aureus* growth. Using fluorescence microscopy, we measured the percent confluence of *S. aureus*growth based of the area of GFP fluorescence within each co-culture chamber as a fraction of the total area of the chamber. *S. aureus*confluence ranged from 19-96%, depending on number of *A. graevenitzii* colonies in the co-culture. As expected, a significant correlation ($r^2 = 0.1355$, ***$p<0.0001$) was observed between the final number of *A. graevenitzii*microcolonies and observed suppression of *S. aureus* growth (Fig. 3D). Interestingly, we did not find a significant correlation ($r^2 = 4.4x10^{-5}$, $p = 0.9297$) between the initial species:species ratio of bacteria loaded and later *S. aureus* growth (Fig. 3E), likely because the rapid doubling time of*S. aureus* overcomes difference in initial bacteria ratio. Thus, the key factor influencing the outcome appears to be the number of*A. graevenitzii* microcolonies present in the co-culture.

In monoculture, *A. graevenitzii* exhibited extensive filamentous growth, formation of new microcolonies and grew to effectively fill the chamber. In contrast, co-culture of *A. graevenitzii* with *S. aureus* resulted in formation of smaller microcolonies with optically dense "core" region bordered by a radial array of relatively short filaments extending outwards into the environment (Fig. 4A,B). These stunted colonies rarely produced secondary colonies (data not shown). We compared the size of exclusion zones formed around microcolonies to the size of the colony "core" and the total colony diameter, which largely reflected the length of the radial filaments extending outwards. These measurements revealed that significantly larger exclusion zones were generated around microcolonies with larger "core" regions ($r^2 = 0.5575$, ***$p < 0.0001$) (Fig. 4C), while colonies with more extensive radial filamentous growth exhibited significantly smaller exclusion zones ($r^2 = 0.2141$, ***$p < 0.0001$) (Fig. 4D). It is likley that the *A. graevenitzii* microcolony morphology observed in co-culture may reflect a state of stress for the bacterium, which might also be related to the formation of exclusion zones. Microcolonies under less stress might exhibit more extensive radial filamentous growth (as observed in monoculture), while colonies under more stress might exhibit restricted filamentous growth, resulting in formation of a larger dense "core" region.

To test whether making more nutrients available affected exclusion zone formation, we compared co-cultures performed in 50 versus 100 μm tall chambers. Relative to *S. aureus* monocultures in each condition, we observed less restriction of *S. aureus* growth in co-culture with *A. graevenitzii* in 100 μm tall (9.8%) chambers compared to 50 μm tall (21.6%) chambers (Fig. S1F). This observation supports our hypothesis that exclusion zone formation occurs in response to competition for nutrients.

*A. graevenitzii* **preferentially grow as clusters in co-culture with *S. aureus***

Spatial clustering of species during co-culture on solid surfaces can provide competative advantages and protect against environmental stresses. In co-culture with *S. aureus* we observed higher numbers of clustered *A. graevenitzii* colonies compared to *A. graevenitzii* monocultures or even co-cultures with *S. cristatus*(Fig. 5A). Measurement of exclusion zones broadly comparing single versus clustered *A. graevenitzii* microcolonies revealed that even very large clusters (> 160 μm diameter) of *A. graevenitzii* did not produce significantly larger exclusion zones compared to single microcolonies (˜20 μm diameter) (Fig. 5B,C).

**Co-culture of *S. aureus* and *A. graevenitzii*modulates innate immune responses**

Given that co-culture with oral flora like *A. graevenitzii*appeared to suppress *S. aureus* growth, we hypoth-

esized that this interaction may also modulate innate immune responses to *S. aureus.* To test this, we measured neutrophil recruitment to co-culture of *S. aureus* and *A. graevenitzii* compared to mono-cultures of *S. aureus* or *A. graevenitzii* alone. As previously reported[17], *S. aureus* mono-cultures induced robust recruitment of neutrophils into the culture chamber (Fig. 6B). Importantly, this response was significantly blunted for co-cultures containing *A. graevenitzii* (Fig. 6B). Interestingly, recruitment of neutrophils to to co-cultures was also lower compared to *A. graevenitzii* mono-cultures (Fig. 6B), suggesting that compounds released during co-culture modifiy the microenvironment and influence neutrophil responses. It is also possible that the two species become less active / more quiscent, and thus they stimulate neutrophils less than exponentially growing cultures.

**Discussion**

We utilized PDMS microfluidic devices to observe microbial mono- and co-cultures of oral isolates in nanoliter volumes. The gas permeability of PDMS facilitates loading of the dead-end chamber through a single channel by applying vacuum, while the coverslip provides optical clarity for imaging approaches and a physical surface on which the microbes can grow. In Actinomyces and Streptococcus co-cultures, we observed formation of a physical "exclusion zones" bordering *Actinomyces graevenitzii* microcolonies with certain species of*Streptococcus* . Interestingly, exclusion zones were also observed with a GFP-expressing strain of *Staphylococcus aureus* , allowing us to perform detailed analysis of exclusion zone formation using automated imaging approaches. These studies supported a model in which exclusion zone formation is triggered by interaction of specific species.

While it appears that the exclusion zone represents a physical space containing no living bacteria, it is unclear whether this is due to physical exclusion for matrix deposition, suppression of proliferation in this area by quorum signaling, or active killing of invading cells by a toxic metabolite. While the formation of exclusion zones only occurred between *Actinomyces graevenitzii* with *S. cristatus, S. salivarius* and *S. aureus* , it is unlikely that the formation of a simple physical barrier explains the interactions. Additionally, the kinetics of *S. aureus* coverage in co-culture with *A. graevenitzii* , which appear to show an initial increase followed by a decrease, suggest that formation of the exclusion zone involves death of existing *S. aureus* cells in that area. Macroscopic co-culture on solid media did not result in any visible cross-inhibition between*A. graevenitizii* and *S. aureus* colonies, highlighting the importance of small volume culture and high-resolution analysis for identification of such interactions. One limitation of the microfluidic chamber design used is the inability to rapidly fix and stain cells to gain a more detailed understanding of their structure, due to the excessive time taken for fixatives and labelling compounds to diffuse through the connecting channel. Either way, this phenomenon provides clear visualization of an antagonistic relationship between competing bacterial species and may provide some insight regarding community structures in the oral cavity.

These interactions are particularly interesting in the context of well-known opportunistic pathogens such as *S. aureus* . Modulation of inflammatory responses in these experiments may be a direct response to compounds released by the bacteria during co-culture, or may simply follow from suppression of *S. aureus* proliferation, which we previously demonstrated to be important for effective neutrophil recruitment[17].

In cases where *S. aureus* is identified in oral lesions, it is often isolated in the company of other opportunistic pathogens such as*Candida albicans[25]* , infections with which are generally associated with loss of microbiome stability. Thus, exclusion of*S. aureus* by *A. graevenitzii* builds on the concept that established and stable commensal communities are important to prevent colonization of a niche by pathogens.

Dissecting the complexity of microbial community structure and its role in the health and disease in the oral cavity remains a focus of ongoing research. Physical and metabolic characteristics facilitate successful colonization of diverse oral surfaces and ongoing survival in this complex and dynamic environment[6]. Many oral microbes have proven challenging to culture, often because they require the presence of one or more speicies in consortia to process specific metabolites[28]. In addition to such relationships, microbial community structure is also dictated by antagonism driven by competition for space and nutrients[29].

Overall, low-volume techniques such as microchambers and droplet-based microfluidics may enhance quorum

sensing and competition for nutrients[9], better mimicking in vivo conditions. The microfluidic technique overcome the limiations of traditional bulk suspension co-culture approaches, which provide limited spatiotemporal information regarding interspecies interactions and often simply result in domination by the faster-growing species.

**Data availablility:** All data presented will be made available by the authors upon request.

**Author Contributions: Fatemeh Jalali:** Data curation (lead), Investigation (lead), Methodology (equal), Visualization (equal), Writing – Original Draft Preparation (supporting).**Felix Ellett:** Formal Analysis (lead), Investigation (supporting), Methodology (equal), Visualization (equal), Supervision (supporting), Writing – Original Draft Preparation (lead), Writing – Review and Editing (equal). **Pooja Balani:** Resources (supporting). **Margaret Duncan:** Conceptualization (equal), Resources (equal), Writing – Review and Editing (equal). **Floyd Dewhirst:** Conceptualization (equal) , Resources (equal), Writing – Review and Editing (equal). **Gary Borisy:** Conceptualization (equal), Resources (equal), Writing – Review and Editing (equal).**Daniel Irimia:** Conceptualization (equal), Project Administration (lead), Supervision (lead), Writing – Review and Editing (equal).

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**References**

1. Paster BJ, Olsen I, Aas JA, Dewhirst FE. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. Periodontol 2000. 2006;42:80-7. Epub 2006/08/26. doi: 10.1111/j.1600-0757.2006.00174.x. PubMed PMID: 16930307.

2. Levin-Sparenberg E, Shin JM, Hastings EM, Freeland M, Segaloff H, Rickard AH, et al. High-throughput quantitative method for assessing coaggregation among oral bacterial species. Lett Appl Microbiol. 2016;63(4):274-81. Epub 2016/07/28. doi: 10.1111/lam.12622. PubMed PMID: 27455031.

3. Postollec F, Norde W, de Vries J, Busscher HJ, van der Mei HC. Interactive forces between co-aggregating and non-co-aggregating oral bacterial pairs. J Dent Res. 2006;85(3):231-4. Epub 2006/02/25. doi: 10.1177/154405910608500305. PubMed PMID: 16498069.

4. Ochiai K, Kurita-Ochiai T, Kamino Y, Ikeda T. Effect of co-aggregation on the pathogenicity of oral bacteria. J Med Microbiol. 1993;39(3):183-90. Epub 1993/09/01. doi: 10.1099/00222615-39-3-183. PubMed PMID: 8366516.

5. Stewart EJ. Growing unculturable bacteria. J Bacteriol. 2012;194(16):4151-60. Epub 2012/06/05. doi: 10.1128/JB.00345-12. PubMed PMID: 22661685; PubMed Central PMCID: PMCPMC3416243.

6. Kolenbrander PE, Palmer RJ, Jr., Periasamy S, Jakubovics NS. Oral multispecies biofilm development and the key role of cell-cell distance. Nat Rev Microbiol. 2010;8(7):471-80. Epub 2010/06/02. doi: 10.1038/nrmicro2381. PubMed PMID: 20514044.

7. Hesselman MC, Odoni DI, Ryback BM, de Groot S, van Heck RG, Keijsers J, et al. A multi-platform flow device for microbial (co-) cultivation and microscopic analysis. PLoS One. 2012;7(5):e36982. Epub 2012/05/19. doi: 10.1371/journal.pone.0036982. PubMed PMID: 22606321; PubMed Central PMCID: PMCPMC3351485.

8. Park J, Kerner A, Burns MA, Lin XN. Microdroplet-enabled highly parallel co-cultivation of microbial communities. PLoS One. 2011;6(2):e17019. Epub 2011/03/03. doi: 10.1371/journal.pone.0017019. PubMed PMID: 21364881; PubMed Central PMCID: PMCPMC3045426.

9. Boedicker JQ, Vincent ME, Ismagilov RF. Microfluidic confinement of single cells of bacteria in small volumes initiates high-density behavior of quorum sensing and growth and reveals its variability. Angew Chem Int Ed Engl. 2009;48(32):5908-11. Epub 2009/07/01. doi: 10.1002/anie.200901550. PubMed PMID: 19565587; PubMed Central PMCID: PMCPMC2748941.

10. Dige I, Raarup MK, Nyengaard JR, Kilian M, Nyvad B. Actinomyces naeslundii in initial dental biofilm formation. Microbiology. 2009;155(Pt 7):2116-26. Epub 2009/05/02. doi: 10.1099/mic.0.027706-0. PubMed PMID: 19406899.

11. Dige I, Nilsson H, Kilian M, Nyvad B. In situ identification of streptococci and other bacteria in initial dental biofilm by confocal laser scanning microscopy and fluorescence in situ hybridization. Eur J Oral Sci. 2007;115(6):459-67. Epub 2007/11/22. doi: 10.1111/j.1600-0722.2007.00494.x. PubMed PMID: 18028053.

12. Nouioui I, Carro L, García-López M, Meier-Kolthoff JP, Woyke T, Kyrpides NC, et al. Genome-based taxonomic classification of the phylum Actinobacteria. Frontiers in microbiology. 2018;9:2007.

13. Bos R, van der Mei HC, Busscher HJ. Co-adhesion of oral microbial pairs under flow in the presence of saliva and lactose. J Dent Res. 1996;75(2):809-15. Epub 1996/02/01. doi: 10.1177/00220345960750021201. PubMed PMID: 8655779.

14. Jakubovics NS, Gill SR, Iobst SE, Vickerman MM, Kolenbrander PE. Regulation of gene expression in a mixed-genus community: stabilized arginine biosynthesis in Streptococcus gordonii by coaggregation with Actinomyces naeslundii. J Bacteriol. 2008;190(10):3646-57. Epub 2008/03/25. doi: 10.1128/JB.00088-08. PubMed PMID: 18359813; PubMed Central PMCID: PMCPMC2395002.

15. He J, Hwang G, Liu Y, Gao L, Kilpatrick-Liverman L, Santarpia P, et al. l-Arginine Modifies the Exopolysaccharide Matrix and Thwarts Streptococcus mutans Outgrowth within Mixed-Species Oral Biofilms. J Bacteriol. 2016;198(19):2651-61. Epub 2016/05/11. doi: 10.1128/JB.00021-16. PubMed PMID: 27161116; PubMed Central PMCID: PMCPMC5019072.

16. Arzmi MH, Alnuaimi AD, Dashper S, Cirillo N, Reynolds EC, McCullough M. Polymicrobial biofilm formation by Candida albicans, Actinomyces naeslundii, and Streptococcus mutans is Candida albicans strain and medium dependent. Med Mycol. 2016;54(8):856-64. Epub 2016/06/30. doi: 10.1093/mmy/myw042. PubMed PMID: 27354487.

17. Ellett F, Jalali F, Marand AL, Jorgensen J, Mutlu BR, Lee J, et al. Microfluidic arenas for war games between neutrophils and microbes. Lab Chip. 2019. Epub 2019/03/14. doi: 10.1039/c8lc01263f. PubMed PMID: 30865740.

18. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin Microbiol Rev. 2015;28(3):603-61. Epub 2015/05/29. doi: 10.1128/CMR.00134-14. PubMed PMID: 26016486; PubMed Central PMCID: PMCPMC4451395.

19. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, et al. The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect Dis. 2005;5(12):751-62. Epub 2005/11/29. doi: 10.1016/S1473-3099(05)70295-4. PubMed PMID: 16310147.

20. Chambers HF, Deleo FR. Waves of resistance: Staphylococcus aureus in the antibiotic era. Nat Rev Microbiol. 2009;7(9):629-41. Epub 2009/08/15. doi: 10.1038/nrmicro2200. PubMed PMID: 19680247; PubMed Central PMCID: PMCPMC2871281.

21. Rigby KM, DeLeo FR. Neutrophils in innate host defense against Staphylococcus aureus infections. Semin Immunopathol. 2012;34(2):237-59. Epub 2011/11/15. doi: 10.1007/s00281-011-0295-3. PubMed PMID:

22080185; PubMed Central PMCID: PMCPMC3271231.

22. Krishna S, Miller LS. Host-pathogen interactions between the skin and Staphylococcus aureus. Curr Opin Microbiol. 2012;15(1):28-35. Epub 2011/12/06. doi: 10.1016/j.mib.2011.11.003. PubMed PMID: 22137885; PubMed Central PMCID: PMCPMC3265682.

23. Siam AR, Hammoudeh M. Staphylococcus aureus triggered reactive arthritis. Ann Rheum Dis. 1995;54(2):131-3. Epub 1995/02/01. PubMed PMID: 7702401; PubMed Central PMCID: PMCPMC1005536.

24. Smith AJ, Jackson MS, Bagg J. The ecology of Staphylococcus species in the oral cavity. J Med Microbiol. 2001;50(11):940-6. Epub 2001/11/09. doi: 10.1099/0022-1317-50-11-940. PubMed PMID: 11699589.

25. Ohman SC, Dahlen G, Moller A, Ohman A. Angular cheilitis: a clinical and microbial study. J Oral Pathol. 1986;15(4):213-7. Epub 1986/04/01. PubMed PMID: 3088236.

26. Iatrou IA, Legakis N, Ioannidou E, Patrikiou A. Anaerobic bacteria in jaw cysts. Br J Oral Maxillofac Surg. 1988;26(1):62-9. Epub 1988/02/01. PubMed PMID: 3422824.

27. Dahlen G, Linde A, Moller AJ, Ohman A. A retrospective study of microbiologic samples from oral mucosal lesions. Oral Surg Oral Med Oral Pathol. 1982;53(3):250-5. Epub 1982/03/01. PubMed PMID: 7038580.

28. Vartoukian SR, Adamowska A, Lawlor M, Moazzez R, Dewhirst FE, Wade WG. In Vitro Cultivation of 'Unculturable' Oral Bacteria, Facilitated by Community Culture and Media Supplementation with Siderophores. PLoS One. 2016;11(1):e0146926. Epub 2016/01/15. doi: 10.1371/journal.pone.0146926. PubMed PMID: 26764907; PubMed Central PMCID: PMCPMC4713201.

29. Nadell CD, Drescher K, Foster KR. Spatial structure, cooperation and competition in biofilms. Nat Rev Microbiol. 2016;14(9):589-600. Epub 2016/07/28. doi: 10.1038/nrmicro.2016.84. PubMed PMID: 27452230.

**Figure Legends**

**Figure 1. A microfluidic device for bacterial nanoliter culture**A) Schematic shows dimensions of microchamber. Each egg-shaped device consists of a central 200 μm diameter, 50 μm high cylindrical microchamber connected to the outer chamber by a 125 μm long, 10 μm wide, 10 μm high connecting channel. B) Depending on how the device is loaded, it can be configured for mono-culture, co-culture, or for studying innate immune responses.

**Figure 2. Species-specific formation of "exclusion zones" in co-cultures of *Actinomyces* with Streptococcal species.** A) Color map shows the results of co-culture experiments with combinations of five strains of actinomyces and three strains of schaalia with seven strains of streptococcus. B) Representative image at 8 hours showing co-culture of *A. naeslundii FCC36* (full yellow arrowheads) and*S. salivarius A64PA33* showing no exclusion zone formation. C) Representative image at 8 hours showing co-culture of *A. graevenitzii FO582* (Empty yellow arrowheads) and *S. salivarius A64PA33* showing formation or large exclusion zones around the *A. graevenitzii* colonies (red dashed circles).

**Figure 3. Exclusion zones are formed in co-cultures of *A. graevenitzii* and *S. aureus.*** A) Time-lapse images show the growth of GFP-expressing *S. aureus* in the presence of *A. graevenitzii FO582* microcolonies. Over 8 hours, *S. aureus*proliferate to fill the chamber (bright green fluorescence), except for the regions bordering *A. graevenitzii* microcolonies (GFP-negative regions). B) Magnified view of co-culture chamber (left) showing growth of exclusion of *S. aureus* from a region containing a cluster of*A. graevenitzii* microcolonies. The cartoon on the right depicts the area of GFP fluorescence measured as a percentage of the chamber. C) Average coverage of microchamber area over time by *S. aureus*growth in presence of different species of *Actinomyces*.Suppression of *S. aureus* growth, corresponding to formation of exclusion zones, was only observed in co-culture with *A. graevenitzii* . Error bars: mean ± SEM. N [?] 5 chambers measured per condition. D) Graph shows the negative correlation between *S. aureus* growth and the number of *A. graevenitzii* microcolonies in each co-culture. Linear regression. Dashed lines are 95% confidence intervals.

N = 152 chambers scored. E) Graph shows no significant correlation between *S. aureus* coverage and the initial ratio of S. aureus: *A. graevenitzii* loaded into each microchamber. N = 178 chambers scored.

**Figure 4. Exclusion zones form around stressed *A. graevenitzii* colonies** . A) Magnified micrograph showing details of*A. graevenitzii FO582* microcolony structure and exclusion zone formation. B) Cartoon depicting colony structure. C) Graph showing the positive correlation between the size of the *A. graevenitzii*microcolony core and the size of the exclusion zone. Linear regression, dashed lines show 95% confidence intervals. N = 75 chambers scored. D) Graph shows negative correlation between the length of *A. graevenitzii* radial filaments and exclusion zone size. Linear regression, dashed lines show 95% confidence intervals. N = 70 chambers scored.

**Figure 5. Clustering of *A. graevenitzii* microcolonies does not increase the exclusion zone in co-culture with *S. aureus*.** A) Graph shows increase in number of clustered *A. graevenitzii FO582* microcolonies compared to monocultures or co-culture with *S. cristatus* . Mean +- SEM. data pooled from at least 2 experiments. B) Cartoon depicts individual versus clustered*A. graevenitzii* microcolonies in co-culture with *S. aureus*measured in (C). Exclusion edge radius measurement is shown in red. C) Scatterplot shows no clear increase in exclusion edge radius, even bordering very large *A. graevenitzii* clusters. N = 161 individual colonies scored. N = 89 clustered colonies scored.

**Figure 6. Modulation of human neutrophil responses to co-cultures of *A. graevenitzii* and *S. aureus*.** A) Representative micrograph showing experimental setup for testing neutrophil recruitment to co-cultures of *A. graevenitzii FO582*and *S. aureus* . B) Bubble plots showing human neutrophil recruitment towards *S. aureus* and *A. graevenitzii* , alone and in co-culture for different loading ratios. The diameter of each bubble represents the number of neutrophils inside each microchamber at the end of experiment. N = 18 chambers scored per condition from 2 independent experiments.

**Supplemental Figure Legends**

**Figure S1. Culture of *S. aureus* in chambers with different volumes in the presence and absence of *A. graevenitzii*.** A-D) Chamber designs with a 200 μm outer chamber and inner chambers of 10 μm (A), 30 μm (B), 50 μm (C), and 100 μm (D). E) Growth of *S. aureus* in chambers of different height, in the presence (upper panels) and absence (lower panels) of *A. graevenitzii FO582* compared to growth observed in the 50 μm design (Fig. 1). Growth of *S. aureus* does not cover the entire area in chambers with heights lower than 50 μm. F) Graph shows measurement of S. aureus coverage in the presence and absence of *A. graevenitzii* in different height chambers. No difference is observed in shallow chambers where *S. aureus* growth is already restricted by the small volume. Comparison of 50 and 100 μm high chambers demonstrated that smaller exclusion zones are generated in larger volume co-cultures. Error bars: mean ± SD, n [?] 5 chambers scored per condition.

**Figure S2. Co-culture of actinomyces and schaalia with streptococcal species in microchambers.** Representative images from microfluidic co-culture experiments, supplemental to Figure 2A. Scale bar: 50 μm.

**Figure S3. Macroscopic co-culture of actinomyces and schaalia species with *S. aureus*.** A) Spot monocultures of actinomyces and schaalia species on BHI agar plates. 2/18 strains failed to grow. B) Co-culture of actinomyces or schaalia species with *Staphylococcus aureus* (Strain SH1000-GFP) did not show macroscopic cross-species inhibition.