REVIEW ARTICLE

Droplet-based Microsystems as Novel Assessment Tools for Oral Microbial Dynamics

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KEYWORDS: Microfluidics; oral cavities; tooth; oral microbiome; nanocultures; unculturable; microbes; bacteria.

Abstract: The human microbiome comprises thousands of microbial species that live in and on the body and play critical roles in human health and disease. Recent findings on the interplay among members of the oral microbiome, defined by a personalized set of microorganisms, have elucidated the role of bacteria and yeasts in oral health and diseases including dental caries, halitosis, and periodontal infections. However, the majority of these studies rely on traditional culturing methods which are limited in their ability of replicating the oral microenvironment, and therefore fail to evaluate key microbial interactions in microbiome dynamics. Novel culturing methods have emerged to address this shortcoming. Here, we reviewed the potential of droplet-based microfluidics as an alternative approach for culturing microorganisms and assessing the oral microbiome dynamics. We discussed the state of the art and recent progress in the field of oral microbiology. Although at its infancy, droplet-based microtechnology presents an interesting potential for elucidating oral microbial dynamics and pathophysiology. We highlight how new findings provided by current microfluidic-based methodologies could advance the investigation of the oral microbiome. We anticipate that our work involving the droplet-based microfluidic technique with a semipermeable membrane will lay the foundations for future microbial dynamics studies and further expand the knowledge of the oral microbiome and its implication in oral health.

1. Introduction

The human microbiome is composed of trillions of microbes and encompasses roughly ninety percent of all cells found in the human body. The microbial consortia of the skin, gut, and respiratory tract work all together to improve the health and well-being of the human host. They digest food, produce essential nutrients and vitamins, prevent the colonization of harmful pathogens, and contribute to the regulation of immune system (Chatzidaki-Livanis et al., 2016; David et al., 2014; He et al., 2015; Kovatcheva-Datchary et al., 2015). However, the understanding of the metabolic functions and the interactions of the human microbiome with its host is limited, which impedes our current ability to treat diseases and improve human health and performance (Dewhirst et al., 2010; Terekhov et al., 2017).

Among others, the oral microbiome is one of the most investigated microbial habitats in the human body (Jenkinson, 2011). It comprises microorganisms inhabiting the oral cavity which includes teeth, saliva, gingival sulcus, attached gingiva, tongue, cheek, lip, hard palate, soft palate, and tonsils. The oral habitat is differentially colonized by a vast array of microorganisms, making a diversified landscape of archaea, fungi, viruses, dominated by bacteria (Dewhirst et al., 2010; He et al., 2015). To date, the oral diversity is estimated to approximately 775 microbial species (Dewhirst et al., 2010; Escapa et al., 2018). Despite such significance, the role and function of the oral microbial ecology as well as its implication to the human host are still unknown.

To address this major knowledge gap, novel technologies have emerged in the recent years to replicate microbial ecology with microsystems that can facilitate the systematic control, cultivation, and analysis of microbial communities for growth conditions, growth patterns and interspecies interactions. Here, we are reviewing current state of the art in the field of oral microbiome, and recent technological advances for deciphering the interplay between oral microbiome and oral health. Far more than the traditional culturing techniques, the microtechnologies are now providing exciting opportunities for designing microsystems and conducting high-throughput studies of interspecies dynamics. This article highlights how droplet-based microfluidics, employed as a novel technique for identifying and investigating new microbial interactions, screen and interrogate microorganisms with bioactive molecules, is now extended to the field of oral microbiology. Ultimately, this critical review lays an interesting perspective regarding the utilization of droplet-based microsystems as an emerging tool for assessing oral microbial dynamics, and bioprospecting or mining the oral microbiome for bioactive metabolites.

2. Deciphering the oral microbiome and its role in oral health and diseases

2.1. Oral bacterial diversity and site-specific composition.

Bacterial colonization of the mouth begins at birth and continues with the development of new colonization niches at the eruption of the primary teeth. The oral environment offers two mechanically distinct surfaces for bacterial colonization: soft tissues of the oral mucosa and hard non-shedding surfaces of the teeth (Zaura et al., 2009). The emergence of hard non-shedding surfaces allows the adhesion, colonization, and accumulation of biomass in the form of dental plaques. Thus, the microorganisms remain constantly in suspension in the saliva, while transitioning to the oral surfaces to form supragingival and subgingival plaques (Dewhirst et al., 2010). The composition of the oral microbiome, therefore, varies based on the physicochemical properties of the microhabitat, and is reshaped from the childhood on through factors including parental or vertical transmission and environmental exposure such as diet (Carlson-Jones et al., 2020; Caselli et al., 2020; Mason et al., 2018; Xu et al., 2015).

A higher microbial diversity is observed on the surfaces of the teeth in contrast to the cheek samples (Zaura et al., 2009). Bacterial genera including *Streptococcus, Granulicatella, Neisseria, Haemophilus, Corynebacterium, Rothia, Actinomyces, Prevotella, Capnocytophaga, Porphyromonas* and *Fusobacterium* are commonly found in varying proportion at the oral sites of healthy individuals. The *Streptococcus* genera is most abundant in areas such as the hard palate, the oral mucosa, and the gingiva while anaerobic bacteria belonging to the genera *Fusobacterium, Actinomyces,* and *Veillonella*, dominate the subgingival plaque (Caselli et al., 2020) (**Fig. 1**). Synergistic relationships among beneficial microbes promote a healthy microbiome, while preventing the upsurge of pathogens. Such competitions in the oral microbiome are commonly associated with phyla including *Bacteroidetes, Fusobacteria, Firmicutes, Proteobacteria,* and *Actinobacteria* (Aas et al., 2005; Chen et al., 2017; Ikeda et al., 2019; Li and Ma, 2020; Xu et al., 2015).

Several studies have determined that the commensal microorganisms maintain a symbiotic equilibrium within their oral microhabitats and the host to form a core microbiome, contributing to the overall oral health (Burcham et al., 2020; Caselli et al., 2020; Zaura et al., 2009). Dysbiosis occurs when the mutualistic relationship among microbiome members, metabolic products, and the host immune system is lost, leading to an overgrowth of pathogenic microbes. Such disturbances within these microhabitats result in an unbalanced oral microbiome promoting a diseased state (Burcham et al., 2020).

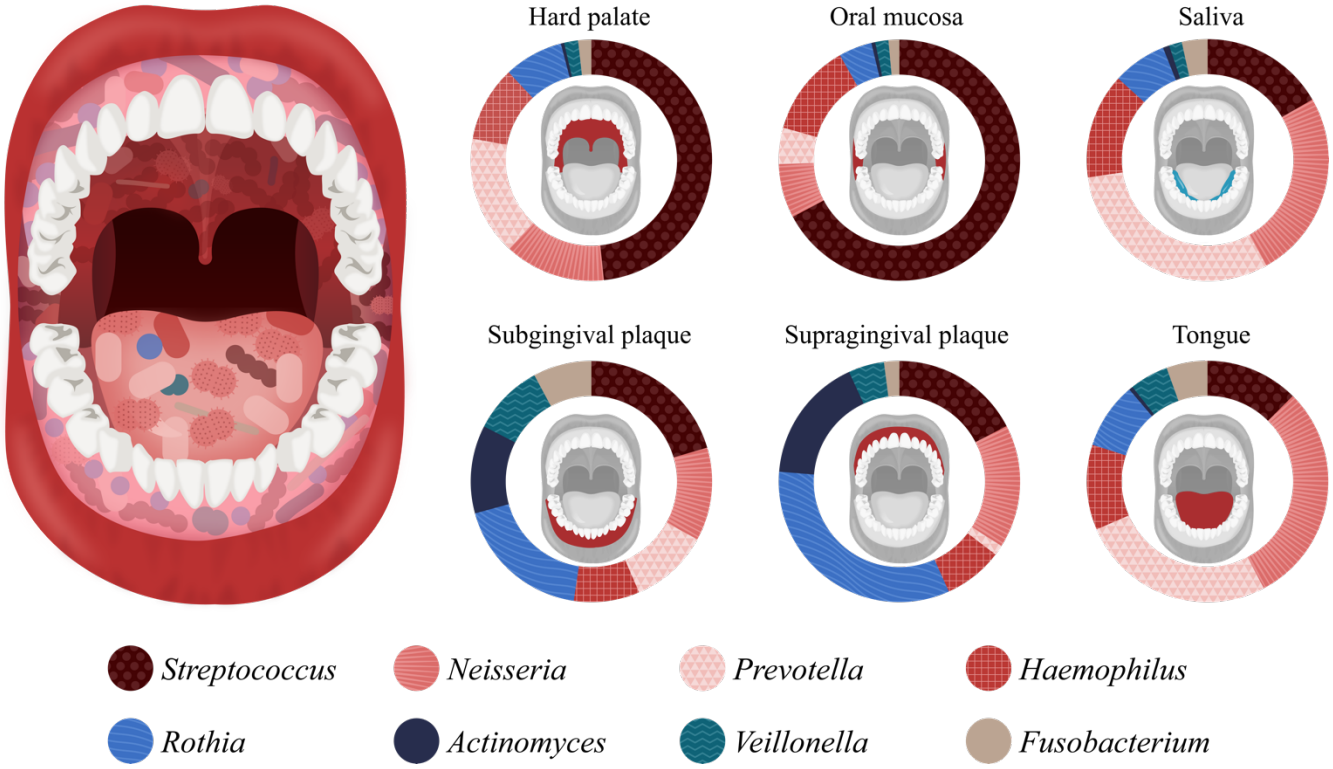

Figure 1. The microbial diversity of the human oral microbiome varies along the oral environments. The *Streptococcus* genera dominates the palate and the mucosa, while the *Rothia*, the *Prevotella* and the *Neisseria* become more abundant in the saliva, the tongue and the dental plaques. Modified from (Caselli et al., 2020).

2.2. The oral microbiome and the diseased state

Balanced composition of commensal microbes that keep pathogens in check is frequently attributed to a healthy oral microbiome. However, disease emerges with a dysbiotic state, under unhealthy equilibrium among microbes and within their microhabitats (Kilian et al., 2016). Microenvironmental and physiological changes can trigger such unbalance within the oral microbiome with adverse impact on the overall human health and well-being (He et al., 2017). For instance, changes in the chemical composition of the mouth, saliva enzyme levels, and increase in macromolecular degradation can provide a suitable environment for the growth and dominance of pathogenic bacteria, such as *Porphyromonas gingivalis, Streptococcus mutans,* and members from the genera *Actinomycetes* and *Lactobacillus,* among others (Barnes et al., 2011; Barnes et al., 2009; He et al., 2015; Marsh, 2006; Miller et al., 2006; Said et al., 2014; Stookey, 2008). Further, the development of medical conditions such as the inflammatory bowel disease can result in an increased proportion of *Bacteroidetes* and a decrease in *Proteobacteria* in the salivary microbiome (Said et al., 2014). Thus, capturing the interplay between the microbes and the host becomes an essential requirement for modulating health beyond the oral environment.

2.2.1 Dental Biofilms as Niches for Microbial Dynamics.

Biofilms are communities of microorganisms enclosed within a self-produced extracellular matrix. The biofilm matrix is composed of extracellular polysaccharides, DNA, and proteins, allowing cells to adhere onto surfaces, grow into microcolonies, and develop stable communities physically separated from their immediate environments (Bowen et al., 2018; Rabin et al., 2015). Within the biofilms, the microorganisms communicate via signaling molecules and interchange genetic materials to acquire new traits; however, the chemical exchange with the biofilm's surroundings remains limited due to the diffusion barrier imposed by the biofilm matrix (Hojo et al., 2009; Rabin et al., 2015). Hence, the biofilm structures permit pathogenic microbes to grow and thrive within defined communities protected from biological, mechanical, and chemical insults (Rabin et al., 2015).

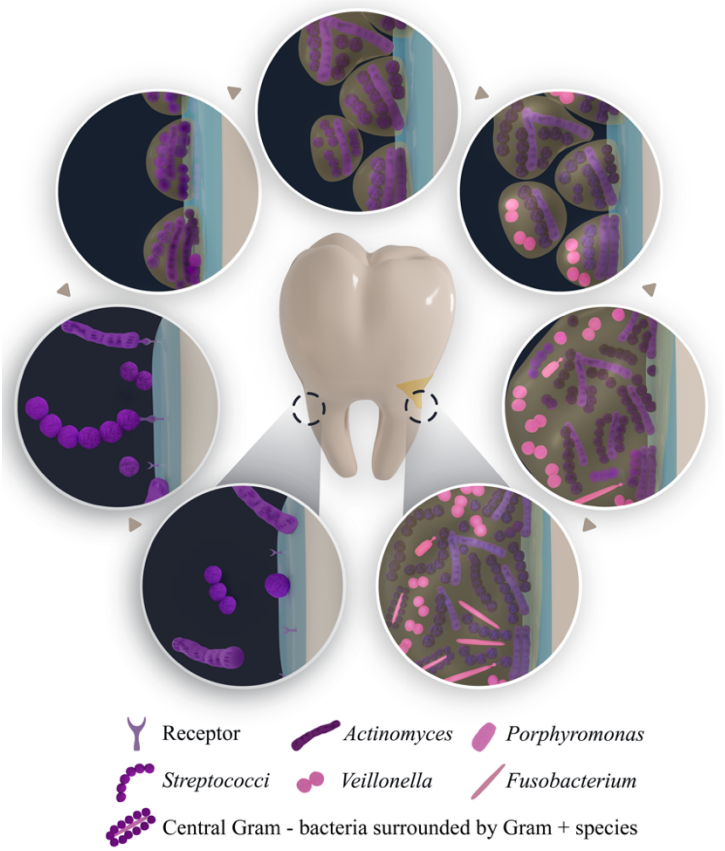

Figure 2: Mechanisms of biofilm formation in oral environment. Gram+ bacteria adhere to the tooth surface by mean of receptors to initiate the dental plaque. Further colonization of the microcolonies by Gram- bacteria lead to the maturation of the dental plaque, and subsequently to oral diseases.

Dental plaques are polyspecies biofilms found on the tooth surface and along the gingival margin (Marsh, 2006; Sbordone and Bortolaia, 2003). Even if the development of plaques does not constitute a health threat, the emergence of opportunistic pathogens and virulent species among the embedded community can cause dysbiosis and lead to a disease state, with an increased tolerance to antimicrobials (Hojo et al., 2009). Bacterial colonization that results in dental plaque occurs in waves, facilitating intra and interspecies communications. An initial colonization occur with planktonic oral microbes, which adhere onto given salivary pellicles on the tooth surface (Rabin et al., 2015; Zijnge et al., 2010). These colonizers, including *Streptococcus*, *Actinomyces*, *Lactobacillus* and occasionally *Candida* species, bind to proteins adsorbed to the tooth enamel by means of receptors on their cell membranes (Cavalcanti et al., 2017; Zijnge et al., 2010). They are then followed by late colonizers, such as *Fusobacterium nucleatum*

and *Porphyromonas gingivalis*. By binding to the early colonizers, the new microbes aggregate into specific partnerships to build another layer of the dental plaque and become a more visible biofilm (**Fig. 2**) (Rabin et al., 2015). The adhesion of the opportunistic pathogens to the plaque increases the virulence of the microbial communities and can accelerate a disease state (Rabin et al., 2015; Ramos-Vivas et al., 2019; Vuotto et al., 2017). Consequently, the eradication of the pathogenic dental biofilms become more challenging, more especially when they are concealed into deep pockets under the gumline (Larsen, 2002; Sedlacek and Walker, 2007; Wright et al., 1997).

2.2.2 Oral Microbial Dynamics and Diseases.

Dental caries and periodontal diseases such as gingivitis and periodontitis are commonly associated with pathogenic dental plaque and biofilms (Bowen et al., 2018; Larsen and Fiehn, 2017; Marsh, 2006; Sbordone and Bortolaia, 2003). The assembly of the extracellular matrix that encloses the dental plaques is powered by microbial metabolism of carbohydrates, which alters the acidity of the microenvironment to the benefit of synergistic pathogenic interactions (Bowen et al., 2018). The accumulation of acid producing and acid-tolerant bacteria such as *Streptococcus mutans,* and those from *Lactobacillus* and *Propionibacterium* genera*,* on the biofilms increase the secretion of acids, in the form of lactic acid for instance, that demineralizes the tooth surface significantly (Bowen et al., 2018; He et al., 2015; Marsh, 2006). The acidophilic species interact synergistically to create an environment conducive to caries development but intolerable to commensal bacteria (Bowen et al., 2018; Marsh, 2006). Thus, a sugar rich diet results in the production of the extracellular matrix of biofilms, which in turn acidifies and demineralizes the tooth tissues, leading to tooth decay and dental caries, the most common oral diseases across all age groups.

In periodontal diseases, the gum, the periodontal membrane, and the alveolar bone are also subjected to destruction and eventual tooth loss due to the accumulation of highly pathogenic supragingival biofilms (Larsen and Fiehn, 2017; Vieira Colombo et al., 2016). High prevalence of pathogenic species in the periodontal site and the biofilm that results as well as its expansion leads to the development of systemic infections (Vieira Colombo et al., 2016). Gingivitis, which is the inflammation of the gums or gingiva, is not a destructive disease in itself. It is associated with the growth of an organized and layered biofilm with a colonization pattern of bacteria arranged by both metabolism and aerotolerance (Nowicki et al., 2018). When the supragingival biofilm is left untreated, it spreads to the periodontal pocket to form a subgingival biofilm, which in turn results in a severe gum infection, a highly inflammatory response followed by bone resorption, referred to as periodontitis (Kilian et al., 2016; Larsen and Fiehn, 2017). In this case again,

bacterial genera including *Actinomycetes, Capnocytophaga, Campylobacter, Eikenella, Fusobacterium,* and *Prevotella* are predominantly associated with the disease progression (Larsen and Fiehn, 2017).

A severe and chronic case of periodontitis can damage soft tissue and destroy dental bone leading to tooth loss (Larsen and Fiehn, 2017). The most common bacteria associated with this chronic inflammatory oral disease are *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia, Bacteroidetes* species*, Eubacterium saphenum, Porphyromonas endodontalis, Prevotella denticola, Parvimonas micra, Peptostreptococcus sp., Filifactor alocis, Desulfobulbus sp., Dialister sp., Synergistetes sp.*, among others (He et al., 2015). Nowicki et al. (2018) reported that the former three species are detected in deep pockets. They increase in proportion in the periodontal pocket and secrete virulent factors that can disturb the healthy microbiome (Nowicki et al., 2018; Vieira Colombo et al., 2016). Additionally, Vieira Colombo et al. (2016) linked species including *Enterobacteria*, *Candida albicans*, *Neisseria* spp., *Pseudomonas aeruginosa*, *Olsenella uli*, *Hafnia alvei*, *Serratia marcescens* and *Filifactor alocis* to periodontal inflammation and tissue destruction. In the presence of such pathogens, commensal bacteria such as *Streptococcus sanguis, Streptococcus uberis* and *Actinomyces viscosus* secrete hydrogen peroxide to protect host environments. The secretion of such metabolites was proven to inhibit the growth of the pathogenic bacteria *Aggregatibacter actinomycetemcomitans* (Nowicki et al., 2018).

Hence, the diversity and relative abundance of oral microbiome play a large role in the appearance and development of oral diseases. Further, the persistence of a dysbiotic state during severe and chronic periodontitis can degenerate into systematic diseases such as diabetes mellitus, bacteremia, endocarditis and other cardiovascular diseases, and tumors (He et al., 2015; Larsen and Fiehn, 2017; Vieira Colombo et al., 2016). It appears, therefore, essential to establish the relationships and specific roles of the oral bacterial communities associated with healthy and dysbiotic oral states. New assessment tools that allow the replication of oral microcosms to investigate the growth patterns and community dynamics accurately and precisely might provide new insights on microbial pathophysiology. Ultimately, the discovery of new paradigms in beneficial interspecies interactions would facilitate strategies to improve oral health.

2.3. Current limitations in growing the unculturable and assessing oral microbiome.

The term “unculturable bacteria” does not portray an inherent inability of bacteria to replicate or grow. Instead, it emphasizes the limitations and flaws in the current culturing techniques for isolating and assessing the growth dynamics of specific microorganisms under laboratory conditions (Browne et al., 2016; He et al., 2015; Stewart, 2012). Thus, the unculturable bacteria refer to challenging microorganisms

that are not yet routinely cultured in the laboratory by means of traditional methods including culture flasks, agar or multiwell plates; and therefore, the knowledge regarding their biology and the role they plays within their respective communities is limited or deeply lacking (Stewart, 2012).

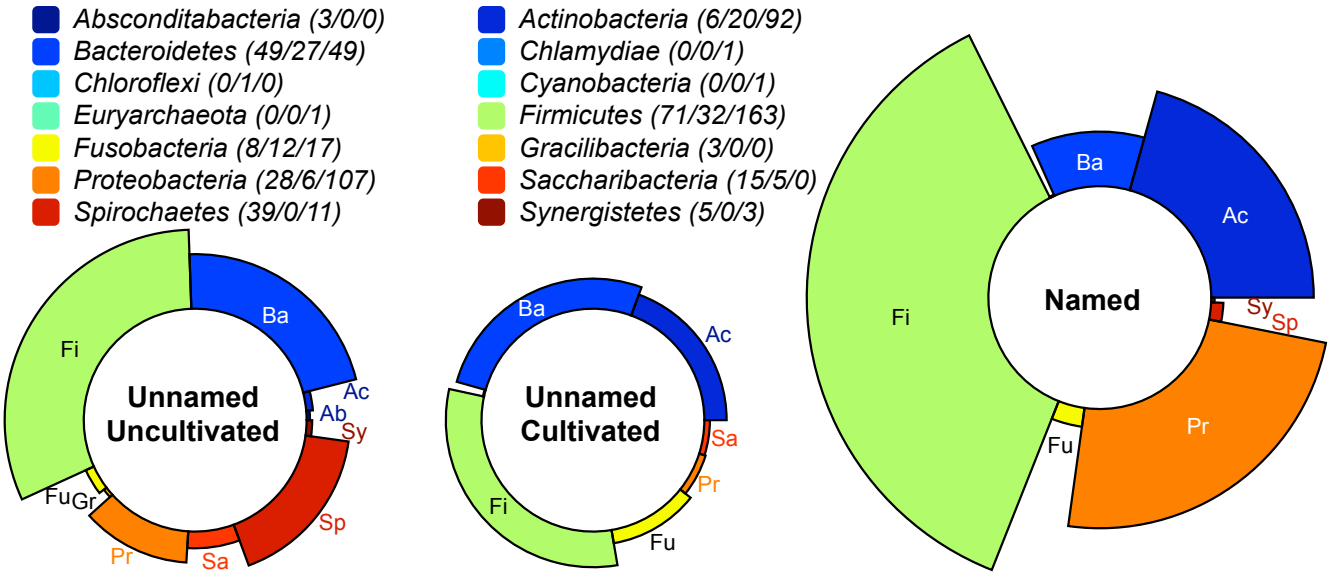

Figure 3. Phylum distribution of 775 microbial species reported in the expanded Human Oral Microbiome Database (eHOMD version 15.22) showing the number and proportion of characterized uncultivated and only known as phylotypes (unnamed uncultivated), cultivated but not characterized yet (unnamed cultivated), and isolates (named). The first two letters for each phylum are reported on the pie chart. The number of species for each group is presented in the following order: (#Unnamed Uncultivated/#Unnamed Cultivated/#Named).

Unfortunately, there exist myriads of such microbial species that have been identified only through molecular techniques but not yet isolated or grown, even with advanced culturing methods (Jenkinson, 2011). The traditional culturing (or culture-dependent) methods coupled with biochemical characterization utilizes either enriched or differentially selective media to grow and identify specific bacterial isolates. These culture media are formulated based on the natural environment of microbes and include specific carbon sources, peptides, and vitamins to promote cell growth under defined pH and oxygen conditions (Ito et al., 2019; Lau et al., 2016; Nagy et al., 2018). Brain Heart Infusion and Lysogeny Broths (LB) are examples of such enriched media, specially optimized for routine growth of a broad variety of bacteria in physiological saline solutions (Barnes et al.; Sambrook and Russell). The growth of a dental sample in such liquid broth, for instance, could allow the election and enrichment of a few species, which could be further selected when streaked onto solid media containing agar. Ultimately, molecular techniques, such as PCR, gel electrophoresis, and high-throughput sequencing are performed for species

identification. However, these synthetic media do not fully recapitulate microbial diversity as they provide elements for synthesizing new cells, and not necessarily the substrates and the conditioning of the native environments (Ajao et al., 2011; Carraro et al., 2011; Wain et al., 2008; Wilkinson et al., 2012; Willems et al., 2013). Thus, important microbial dynamics are lost because some species occur in low proportions due to the inability to properly replicate or expose cells to essential aspects of their natural microenvironments (Stewart, 2012). Methods such as co-cultivations or supplementation of additives capable of simulating the native microbial environments, such as artificial saliva, are still being attempted to improve microbial culturability (D'Onofrio et al., 2010; Kaeberlein et al., 2002; Strandwitz et al., 2019).

Despite the challenge for mimicking the environmental conditions and interactions between the microbiome and its host, the culture-dependent methods are still the gold standard for studying culturable microbes routinely (Browne et al., 2016; Pozhitkov et al., 2011; Stewart, 2012; Vartoukian et al., 2016). They have enabled the cultivation of about two third of the total estimate of oral bacterial species (Thompson et al., 2015; Vartoukian et al., 2016). The expanded Human Oral Microbiome Database (eHOMD) reports that 30% of the 775 oral microbial species are still uncultivated and only identified through their genomic sequences (Escapa et al., 2018). These proportion of unculturable species varies greatly among the phyla, and is more particularly underrepresented among the *Spirochaetes*, where more than 70% species remains poorly cultivated and understudied (**Fig. 3**).

Recent advances in culture-independent methods, such as molecular techniques involving high-throughput sequencing, metabolomics, proteomics, checkerboard DNA-DNA hybridization, have shed more light on microbial diversity without the need for culturing each member of the given communities (Ahn et al., 2011; Chhour et al., 2005; Dowd et al., 2008; He et al., 2015; Jenkinson, 2011; Keijser et al., 2008; Lazarevic et al., 2009; Nasidze et al., 2009; Preza et al., 2009). Because these techniques do not preserve a viable microbial community, it become extremely challenging to recapitulate the causal relationships among the microbial populations and with their host environments (Ito et al., 2019; Shirali Pandya et al., 2017). And this major limitation must still be overcome for fully characterizing the oral microbiome.

It is therefore irrefutable that platforms that combine both culture -dependent and -independent methods would provide a significant advantage for assessing new microbial dynamics (Ito et al., 2019; Lau et al., 2016; Shirali Pandya et al., 2017). Developing novel technologies to 1) isolate, 2) improve the culturability of new species or of those naturally appearing in low abundance, and 3) characterize their

interplay within the oral microbiome with named or known microbes could offer new insights and elucidate oral diseases and health (Al-Awadhi et al., 2013; Shirali Pandya et al., 2017). In this regard, ultra-high-throughput droplet-based microtechnologies are emerging in the field of oral microbiology to carefully mimic the native microenvironment, screen and characterize disease-associated oral microbial communities at a species-level resolution.

3. Advantages and Unique Potentials of Droplet-based Microfluidics for Studying Microbes

A high-throughput technology allows a faster production and more efficient screening or testing of thousands and millions of samples. Microfluidics is an example of a promising high-throughput microtechnology that has recently emerged to study microbial communities *in vitro* and to model their interactions at cellular dimensions. The technology enables the manipulation of small amounts of fluid or liquid suspensions at micro-, nano-, and even femto-scales (around 10^{-9} to 10^{-18} L) to generate emulsions and single or multicore microcapsules. The emulsions can be synthesized in batch by stirring two immiscible phases using sonication or a high-speed rotating mixer (**Fig. 4a**), and by controlling fluid flow using miniaturized integrated channels (**Fig. 4b**) or a flow-focusing microfluidic device (**Fig. 4c,d**) (Dhand et al., 2021; Shang et al., 2017; Whitesides, 2006). Thanks to advances in microfabrication, single-cell analyses using microfluidics have become accessible and reproducible. The design of controlled experimental configurations with low volume of reagents and the automated high-throughput nature of the assays is both efficient and cost-effective (Leung et al., 2012).

A method that has attracted major attentions in the field of microbiology for its pioneering applications and advantages over previous culturing and assessment techniques is the droplet-microfluidics. Droplet microfluidics allow for the continuous flow of discrete microemulsions in confined environments all while maintaining precise control of droplet properties such as temperature, pH, osmotic pressure, content, position, and stability (Mashaghi et al., 2016; Shang et al., 2017). Microorganisms are resuspended in liquid media and encapsulated into droplets or microgels. The smaller volumes of nutrients in the droplets permit a faster generation of metabolites and molecules secreted by the microorganisms (Boedicker et al., 2009). The encapsulation of microbial suspensions facilitates the independent manipulation and interrogation of cells within defined communities (Shang et al., 2017). The ultra-high encapsulation and cultivation of individual microbial isolates can be exploited to uncover the relative abundance and diversity found in various communities. This process accelerated the early detection of cells occurring at low-density and of rare slow-growing species from complex environments, an issue commonly unsolved with traditional and classical culturing methods (Kaminski et al., 2016; Shang et al., 2017).

The ability to analyze individual cells in the miniaturized cultures has provided increased sensitivity and high-resolution information on cellular reactions, contributing to understand the heterogeneity and physiological stages of defined microbial populations (White et al., 2011). Because each cell is harnessed within its own microcompartment, nutrient competition is eliminated, and the physico-chemical and chemical interactions amongst microorganisms can easily be decoupled (Niepa et al., 2016). Millions of droplets, collected in a short time frame, can be screened, sorted, and characterized for genotypic and phenotypic variances (Kaminski et al., 2016; Shang et al., 2017). The high parallelization of experimental conditions provided by these microsystems is far superior than that achieved with the traditional culturing methods, mitigating past limitations for species characterization and downstream analytical processes (Liu and Zhu, 2020). By providing arrays of controlled microenvironments, microfluidics coupled with downstream analyses such as metagenomics have proven successful for culturing bacterial strains previously "unculturable", and characterizing novel species of commercial and therapeutic interests (Zhang et al., 2017).

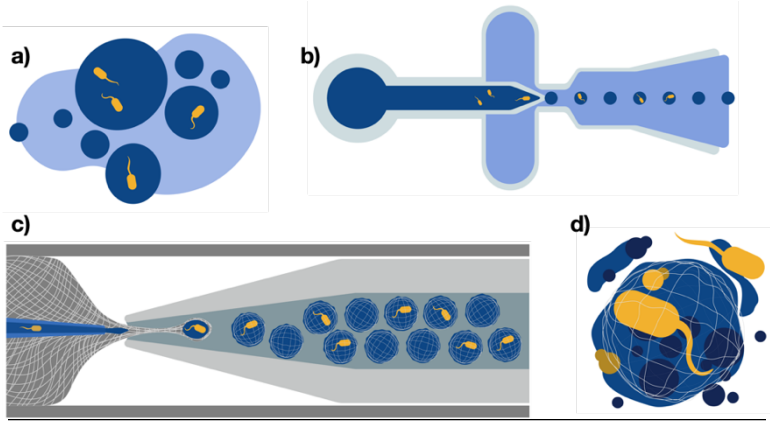

Figure 4: Droplet-based techniques for microbial encapsulation. **(a)** Bulk emulsions are generated using phase separation by mechanical agitation and stirring. **(b)** Single emulsions are generated using PDMS-based microfluidic devices. The device is designed on a silicon wafer via soft-lithography techniques and the PDMS is casted and cured on the wafer to print the device. An oil and water phase containing a surfactant enable the high-throughput generation of the droplets encapsulating microorganisms. **(c)** Flow-focusing microfluidic device allows the generation of double emulsions. The device is created using square and circular capillaries shaped and spaced precisely to flow three phases and generate water-in-oil-in-water emulsions. **(d)** The middle phase of the nanocultures containing microorganisms is made of polymeric shell, which crosslinks during the incubation.

3.1. Microfluidics technologies for assessing microbial dynamics and detecting microbial functions

Several studies have implemented the droplet-based microfluidic approach to assess microbial communities, isolate and identify microorganisms, investigate microbial physiology and cell-cell interactions, and measure antimicrobial susceptibility (Kaminski et al., 2016; Leung et al., 2012; Pratt et al., 2019; Terekhov et al., 2017). For instance, Yaginuma et al. (2019) utilized droplet-based microfluidics for coculturing reporter mammalian cells and yeast cells in water-in-oil droplets for high throughput identification of functional peptide ligands against G-protein coupled receptors. Following the binding of the secreted peptide ligand to the reporter cells, the droplets were sorted to identify the relevant yeast using

sequencing (Yaginuma et al., 2019). In another application of microfluidic platform developed by Zhang et al. (2017), the droplets were used to precisely isolate single cells from a mixture microorganisms of microorganisms composed of microalgal and yeast cells. Studies, such as those previously mentioned, combined the microfluidic platforms with various molecular techniques, such as high throughput DNA sequencing, mass spectrometry, quantitative PCR, to examine cell functions at the molecular level and ultimately identify individual cells secreting specific molecules (Hsu et al., 2019; Terekhov et al., 2017; Villa et al., 2019; White et al., 2011; Yaginuma et al., 2019; Zhang et al., 2017). Although most of these studies showcases how microfluidic droplets can be used to elucidate complex communities, the methods and expected findings could be extrapolated to the study of the oral microbiome (Villa et al., 2019).

In many instances, droplet-microfluidics heavily rely on imaging platforms to identify microbial functions. (Hsu et al., 2019; Huang et al., 2015; Leung et al., 2012; Niepa et al., 2016; Park et al., 2011; Terekhov et al., 2017). For instance, Park et al. (2011) uses a simple microfluidic platform in the co-cultivation of microbial communities to discern direct symbiotic relationships within a synthetic model system consisting of cross-feeding *E. coli* mutants. This study exemplifies the high parallel nature of these devices and showcases how they can be used to cultivate and isolate distinct microbes and show the extent of their interactions (Park et al., 2011). On the other hand, Hsu et al. (2019)'s developed a microfluidic platform, the Microbial Interaction Network Inference or MINI-Drop, that is coupled with fluorescent microscopy to rapidly infer microbial interactions based on the absolute abundance of particular strains. The group examined the growth dynamics of RFP-labeled *E. coli*, CFP-labeled *E. coli* methionine auxotroph, and YFP-labeled *S. Typhimurium* deficient in lactose metabolism in the presence of minimal media. Because of the reliance of fluorescent labels, the study of microbial dynamics using the fluorescent imaging is limited to a three-member community expressing distinguishable fluorescence. Nonetheless, the automated cell counting platform provides the basis for scaling up the approach for the study of complex communities, such these found in the oral microbiome. This might require machine learning algorithms for assessing microbial dynamics not only based on parameters such as fluorescence, but also based on size and motility functions such as velocity and mean-square displacement. Coupled with some high-throughput and automated imaging techniques, these algorithms could provide new insights on oral microbiome dynamics, which includes 775 members.

A major advantage of the droplet-based microfluidics is that the technique had advanced the mining or bioprospection of individual microbe to systematically explore, screen, and extract beneficial metabolites secreted by live-biotherapeutics. For instance, novel microbial species with higher enzymatic

activities have been isolated, providing novel opportunities for exploiting the innate abilities of microbes to produce bioactive metabolites (Gonçalves et al., 2020; He et al., 2019; Kaminski et al., 2016; Najah et al., 2014). With the spread of drug-resistant microorganism, the bioprospection of bioactive compounds from newly characterized species is urging for identify new antibiotics. (Terekhov et al., 2020). And the microfluidics allow to discern new antibiotic producers through the analysis of antimicrobial susceptibility in droplets.

Mahler et al. (2018) demonstrate the use of this microfluidic platform in the high throughput screening and detection of antimicrobial compounds produced in droplets from single cells by observing the survival of reporter cells. The study exhibited the production of secondary metabolites in the single-cell droplets. Specifically, in droplets containing *Streptomyces griseus* cultures, the antimicrobial secondary metabolite streptomycin was produced (Mahler et al., 2018). Mahler et al. (2019) continued in a later study to demonstrate the ability to discover novel antimicrobial producers using droplet microfluidics. Through the screening of reporter strains *Escherichia coli* and *Bacillus subtilis*, which represented Gram-positive and Gram-negative bacteria respectively, the study revealed many antibiotic producing isolates, some of which possibly represented a new genus (Mahler et al., 2019). This is only one approach to the study of novel antibiotic producers. Different reports demonstrate a range of methods in the detection of antimicrobial producing strains (Kulesa et al., 2018; Miller et al., 2012; Shembekar, 2018; Terekhov et al., 2020). Wippold et al. (2020) described a droplet microfluidic system, PRESCIENT (Platform for the Rapid Evaluation of antibody SucCess using Integrated microfluidics ENabled Technology), that first aids in producing antibodies from a single hybridoma cell and then characterizing the antibodies produced from the cell that indeed neutralize infectious agents. These studies work to alleviate the growing global issue of antibiotic resistance and support the effort for designing new therapies against viruses and opportunistic pathogens. Overall, such advances in antimicrobial and antibiotic discoveries demonstrate the versatility of droplet-microfluidics in bioprospection studies offering innovative and high-efficient approaches to identify and efficiently select microorganisms with valuable metabolic capabilities for the industrial and health sectors.

3.2. Recent Advances in the Study of using Microfluidic Droplet Emulsions

The use of droplet-based microfluidics for studying the oral microbiome is in its infancy. Very few works have employed this novel technique to identify key oral microbial dynamics. These exploratory studies focus on tool development and proof-of-feasibility for assessing the oral microbiome. For instance, Leung et al. (2012) presented a programmable droplet-based microfluidic device that successfully analyzed

common laboratory strains and microbial communities. The device uses a multipurpose microvalve technology to program the release of nanoliter volume of reagents into 95 microchambers (**Fig. 5**). The platform takes advantage of the wetting properties of the microfluidic channels to control the flow of the reagents into the chambers. Thus, microbial suspensions could be introduced to deliver single-cells or create a defined community in each chamber with precision. This device enables a flow-routing control to interrogate the resulting community or manipulate microbial functions. The final product of the reactions is retrieved from the chip into microfuge tubes through an integrated elution nozzle with software-assisted automation. Downstream analyses including single microbe culturing and sorting, PCR-based genotyping, whole genome amplification can be performed.

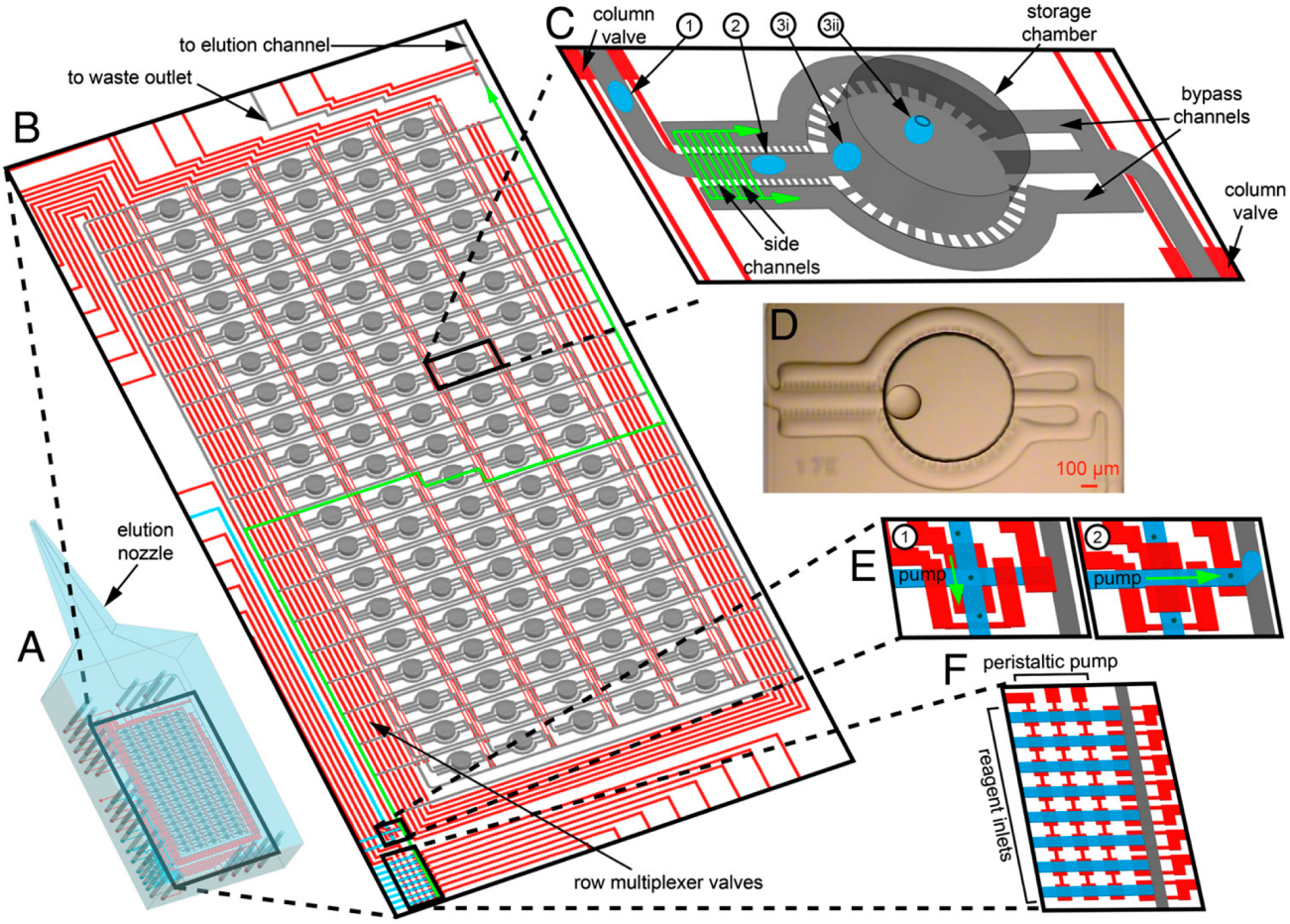

Figure 5. **(A)** Programmable droplet-based microfluidic platform developed by Leung et al. (2012). (A) Elution nozzle enables the collection of droplets. **(B)** Multipurpose microvalve technology to program the release of nanoliter volume of reagents into 95 microchambers. **(C)** A single chamber allowing the reaction is shown. (1-3) The platform takes advantage of the wetting properties of the microfluidic channels to control the flow of the reagents into the chambers.

(D) Representative image of a 2.7 nL water droplet in a chamber. (E) Cell-sorting module and (F) the Reagent-metering module are shown. Reproduced with permission (Leung et al., 2012).

The platform was used to investigate the genotypic properties of the individual bacterial cells recovered from biofilm samples isolated from a human oral cavity. An oral swab was resuspended in phosphate-buffered saline solution for cell cultivation in the microfluidics platform. After cultivation, the samples were sorted, and the microbial population were sequenced to identify oral microbiome genera (Leung et al., 2012). The results corroborated with oral microbial community isolated from human previously reported as culturable. The findings include the *Capnocytophaga* and *Flavobacterium* within the *Bacteroidetes, Corynebacterium, Rothia, Kocuria* and *Actinomyces* within the *Actinobacteria, Fusobacterium* within the *Fusobacteria, Clostridium* and *Streptococcus* within the *Firmicutes*. Also, novel species found in low abundance and now referred to as the *Saccharibacteria* was detected during the analysis of the droplets (Leung et al., 2012).

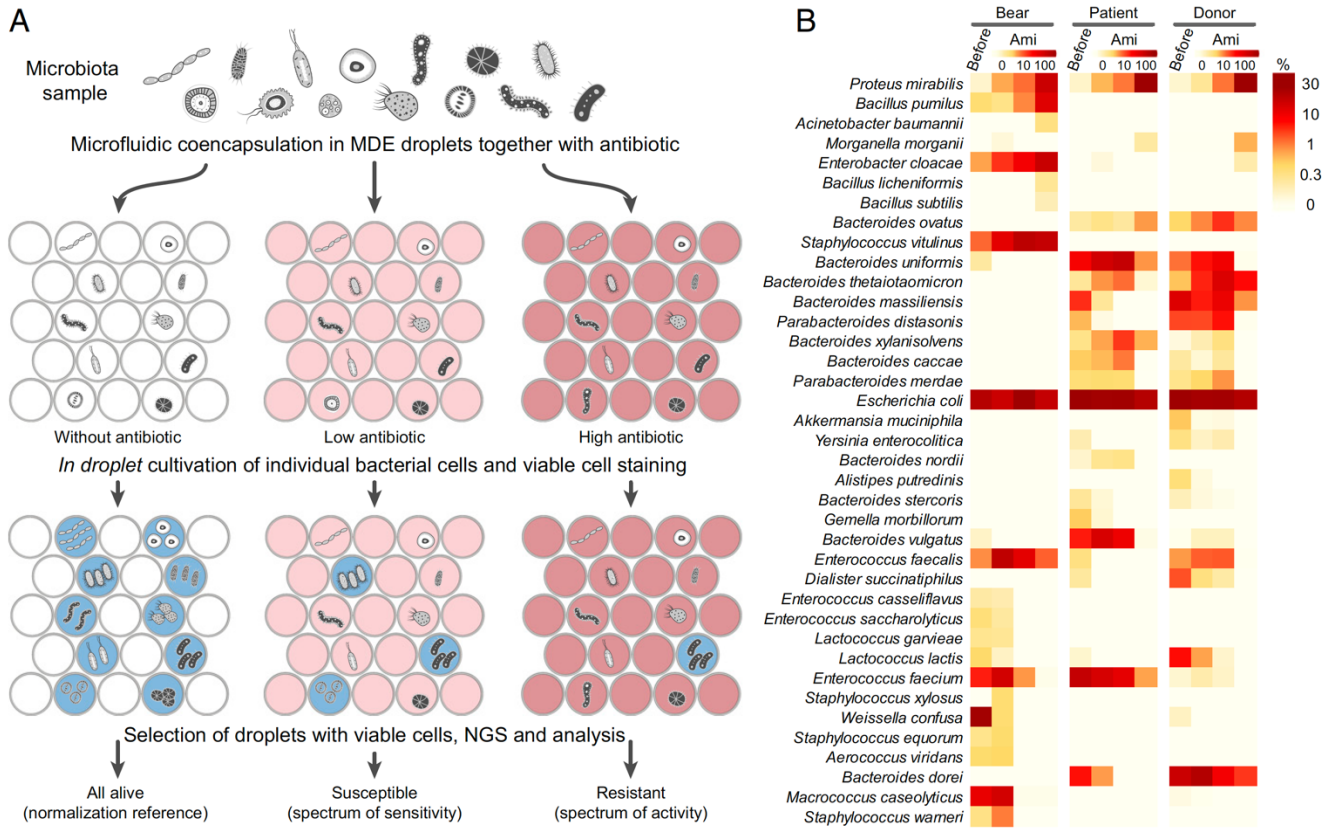

Figure 6. (A) Microfluidic Droplet Emulsion coupled with Fluorescence-Activated Cell Sorting (MDE-FACS) served to screen oral microbiota members for the ability to secrete bioactive molecules. Antibiotic Amicoumacin (Ami) was isolated and tested on microorganisms to evaluate their susceptibility and resistance to the drug. (B) The shift in the diversity of the microorganisms found in the Siberian brown bear oral cavity, as well as the fecal microbiota of a healthy human, and

a human with colitis following treated with 0, 10, 100 μg/mL Ami is shown. Reproduced with permission (Terekhov et al., 2018)

Terekhov et al. (2017) developed an ultrahigh-throughput (uHT) microfluidic droplet platform to cultivate single cells, and screen microbial diversity and functionality in environmental samples. A flow-focusing microfluidic chip was used to generate nanoliter volume microbial cultures as water-in-oil-in-water double emulsions, with mineral oil as the shell of the compartment. The device was coupled with Fluorescence-Activated Cell Sorting (FACS), which selected for microbes, catalyzing a specific reaction resulting in the expression of the fluorescent marker. The emulsions containing the microorganisms of interest (the activators) were collected to determine their genotypic, phenotypic, and structural functionalities using downstream analyses including next-generation sequencing and liquid chromatography-mass spectrometry.

The technology was applied to select for human oral microbiota with inhibitory activity against GFP expressing *Staphylococcus aureus*. Initially, the pathogens were labeled with sCy5 to stain the live and dead cells into a red fluorescence. Then, the *S. aureus* cells were co-encapsulated in the double emulsions and co-cultivated with the human oral microbial isolates. Further Calcein Violet AM staining of the droplet containing any live cells resulted in a blue fluorescence that was captured using FACS. Therefore, the droplets containing live GFP-expressing *S. aureus* emitted both green and blue fluorescences, and those with the dead *S. aureus* emitted a red fluorescence alone. Any droplets with the live oral microbial isolates capable of inhibiting or killing the pathogen emitted a blue –from the Calcein Violet AM– and red fluorescence because of the presence of the dead *S. aureus*. Using the MDE-FACS technique, beneficial oral microbes from the *Streptococcus* genus, and more particularly *Streptococcus oralis,* were isolated and identified (Terekhov et al., 2017).

Terekhov et al. (2018) extended the application of the MDE-FACS platform to the bioprospecting of bioactive compounds secreted by oral microbial communities from the Siberian brown bear. There again, the microbial isolates were co-encapsulated with GFP-labeled *S. aureus* at a rate of 30,000 droplets per second. Following cultivation, droplets displaying a reduction in GFP expression of *S. aureus* and high antagonistic activity with beneficial species were sorted based on the blue fluorescence associated with Calcein Violet AM metabolism. Thus, deep functional profiling was performed to identify bacteria with inhibitory activity against the pathogen, which included *Enterococcus casseliflavus*, *Weissella confusa*, and *Bacillus pumilus*. However, a *B. pumilus* strain producing antibiotic Amicoumacin (Ami) was selected

for its strong anti-*Staphylococcus* activity. The strain was analyzed to understand the bacterium ability to secrete and resist to the self-produced antibiotic. The antimicrobial activity of the new compound was tested in the droplets against *S. aureus*, and a wide range of microorganisms found in the Siberian brown bear oral cavity, as well as the fecal microbiota of a healthy human, and a human with colitis (**Fig. 6**) (Terekhov et al., 2018).

Furthermore, Terekhov et al. (2020) optimized the growth conditions of *B. pumilus* in light of proteomic and metabolomic analyses to improve the secretion of Ami. A medium with high carbohydrate content and calcium carbonate was developed to scale up Ami production under increased aeration, which facilitated the testing of the antibiotic efficiency against a wide range of pathogens including *H. pylori*, *Vibrio*, *Enterococcus* and *S. aureus* strains. The minimum inhibitory concentration (MIC) of Ami against clinical isolates was measured in the droplets and corroborated with conventional testing methods. The results suggested that the antibiotic was less potent against some wild type *E. coli* strains. In fact, Ami was found to lose his efficacy in physiological conditions because of the denaturation into a more inactive form, suggesting that additional work is needed to improve the stability and effectiveness of the antibiotic. Still, these studies establish the potential of the droplet-based microfluidic for: 1) Discovering antimicrobials from oral source; and 2) Evaluating the antibacterial spectrum of the compound, which was found to alter the diversity of oral and fecal microbiomes (Terekhov et al., 2020; Terekhov et al., 2018; Terekhov et al., 2017).

Overall, the studies by Leung et al. (2012) and Terekhov et al. (2020) denote significant progress in the study of oral microbial dynamics using microfluidic droplet emulsions. They demonstrate how the current advances in microfluidics have enabled the design complex microsystems, that when coupled with downstream techniques advanced microscopy and omics-studies, can help profile the oral microbiota, isolate bioactive metabolites, and develop novel antibiotics from isolated microbial species. However, the discovery of new microorganisms, especially those known as unculturable, might require the design of much robust microsystems, which are capable of growing cells *in situ*. To this end, the polymeric microsystems could present attractive features which are worth exploring.

4. Outlooks on polymeric droplet-based microsystems for assessing oral microbial dynamics.

The high throughput droplet-based microfluidic approach is a relatively new in the field of oral microbiology but has a countless range of applications for assessing microbial communities in defined microenvironments. The technique could help create artificial microniches for recapitulating microbiome

dynamics, cultivating microbes *in situ*, and delivering live biotherapeutics and bioactive compounds relevant to oral health. However, several challenges need to be overcome to meet these goals. For instance, the platforms need to be specifically designed to suit the needs of the study, which may in turn be both time consuming and costly. To achieve downstream processes required for microbial identification, the microfluidic platforms need to be paired with several analytical techniques. Whether the goal is to sort droplets, observe and analyze interactions among microbial communities, or encapsulate and incubate individual cells over a long period of time, the microfluidic device must be tailored to such demands. Most droplet-based microfluidic devices utilize oil-based emulsions, which are fragile and difficult to manipulate outside of an aqueous environment such as to grow cells *in situ*.

A possible solution to these obstacles is the use of a polymeric shell exhibiting a selective permeability to essential microbial growth factors and metabolites (Niepa et al., 2016). In the past, semipermeable micromachined platforms have been used to culture microbes in a version of their natural environment and receive nutrients and conditions promoting their growth. For instance, (Nichols et al., 2010) created a diffusion chamber that allowed them to grow previously unculturable bacteria in the laboratory. Diffusion-chamber based approaches were also used to create an isolation chip (iChip) composed of several hundred of miniature diffusion chambers each inoculated with a single environmental cell (Bollmann et al., 2007). Such microsystems have facilitated the cultivation of hundreds of taxonomic units of soil and marine microbes, which were previously considered unculturable, setting the precedent for growing cells in their native environments by means of semi-permeable confinements.

Inspired by the iChip, polymeric droplet-based microchambers were developed for exploring cell-cell communication and interkingdom interactions across the shell of a selectively permeable and mechanically robust microsystem (Kim et al., 2017; Niepa et al., 2016). Two pathogenic members of the oral microbiota were considered: *S. mutans*, known to catalyze sucrose into the monosaccharide glucose and fructose during the production of the glycans making up its biofilm, and *C. albicans*, utilizing glucose as the main carbon source. The common association of both pathogens with dental caries led to the hypothesis that a synergistic interaction between the yeast and the bacterium might affect the disease outcome. Thus, Kim et al. (2017) used a flow-focusing microfluidic device to generate spherical nanoliter volume cultures harboring *S. mutans* in polydimethylsiloxane (PDMS) membrane, known as the nanocultures. Initially, the PDMS mixture was supplemented with silicon oil to produce the shell of the nanocultures, which were allowed to crosslink at 37°C in the presence of platinum catalyst. By

sequestering *S. mutans* in a polymeric shell surrounded by conditioned media resulting from the co-cultivation of both pathogens, Kim et al. (2017) demonstrated that the diffusion of secondary metabolites generating by *C. albicans* into the nanocultures promoted the growth of other pathogenic species (**Fig. 7**). Using the selectivity of the nanoculture shell, they determined that farnesol secreted by *C. albicans* stimulated *S. mutans* biofilm by inducing (by 4-fold) the expression of *gtfB*, a gene responsible for the production of glucosyltransferases (Gtf) and extracellular glucans (Kim et al., 2017). Meanwhile, the overproduction of farnesol was found to eradicate *S. mutans*, suggesting that farnesol was appropriately secreted to modulate the growth of *S. mutans* and supply glucose in return. Although there is not an extensive number of investigations pertaining to the application of the transport properties of the microcapsules for studying the oral cavity, the study provides a new perspective for decoupling the chemical and physical interactions of microorganisms while exploring community dynamics. Optimizing this high-throughput platforms could provide new insights on oral microbial pathophysiology and on the design of tools for stimulating the growth of slow-growing organisms *in situ*.

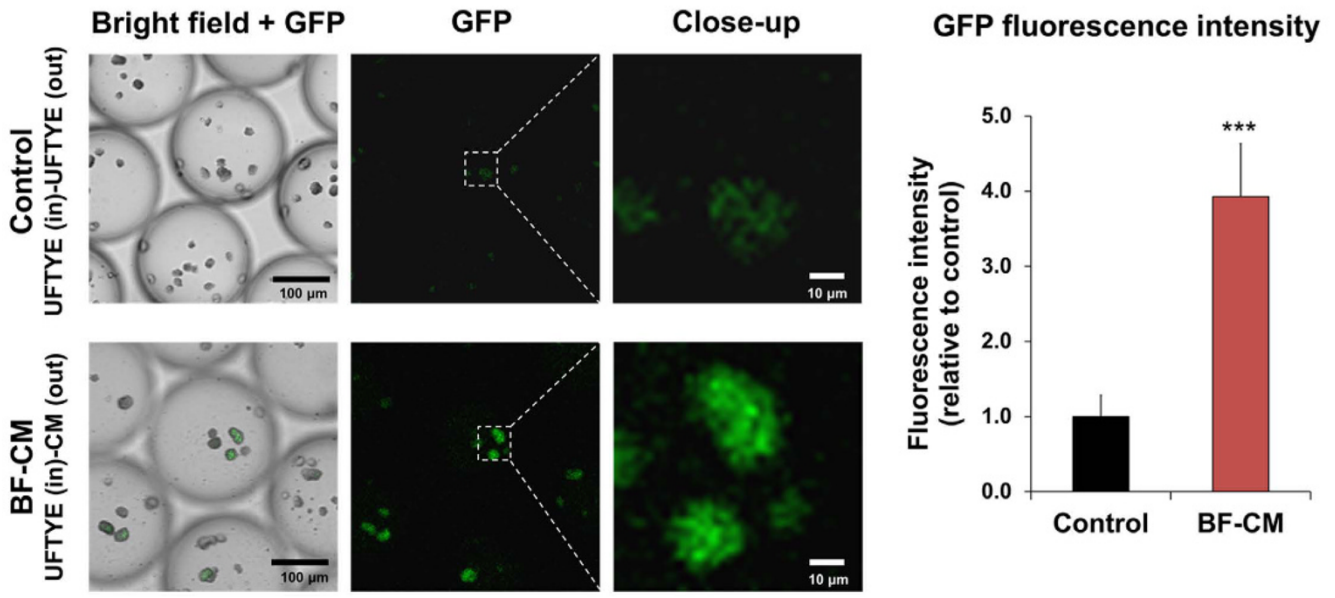

Figure 7. Microcolony formation by *S. mutans* in the presence and absence of conditioned media from the bacterial-fungal (BF-CM) biofilms. *S. mutans* were encapsulating in the selectively permeable nanocultures. The diffusion of the farnesol across the shell of the nanoculture induced GFP expression by 4 fold in a reporter strain under the control of *gtfB* promoter; *PgtfB::gfp*. Reproduced with permission (Kim et al., 2017)

With the advances in functional materials, new membranes are being developed to provide new tools and artificial microhabitats for the study and manipulation of complex microbial consortia (Wondraczek

et al., 2019). Building upon the advantages of the droplet-based microfluidics such as low cost and easy manipulations, the new polymeric mixtures are incorporated in the design of emulsion droplets for rapid isolation and long-term incubation of cell growth in environments requiring high shear and mechanical stress.

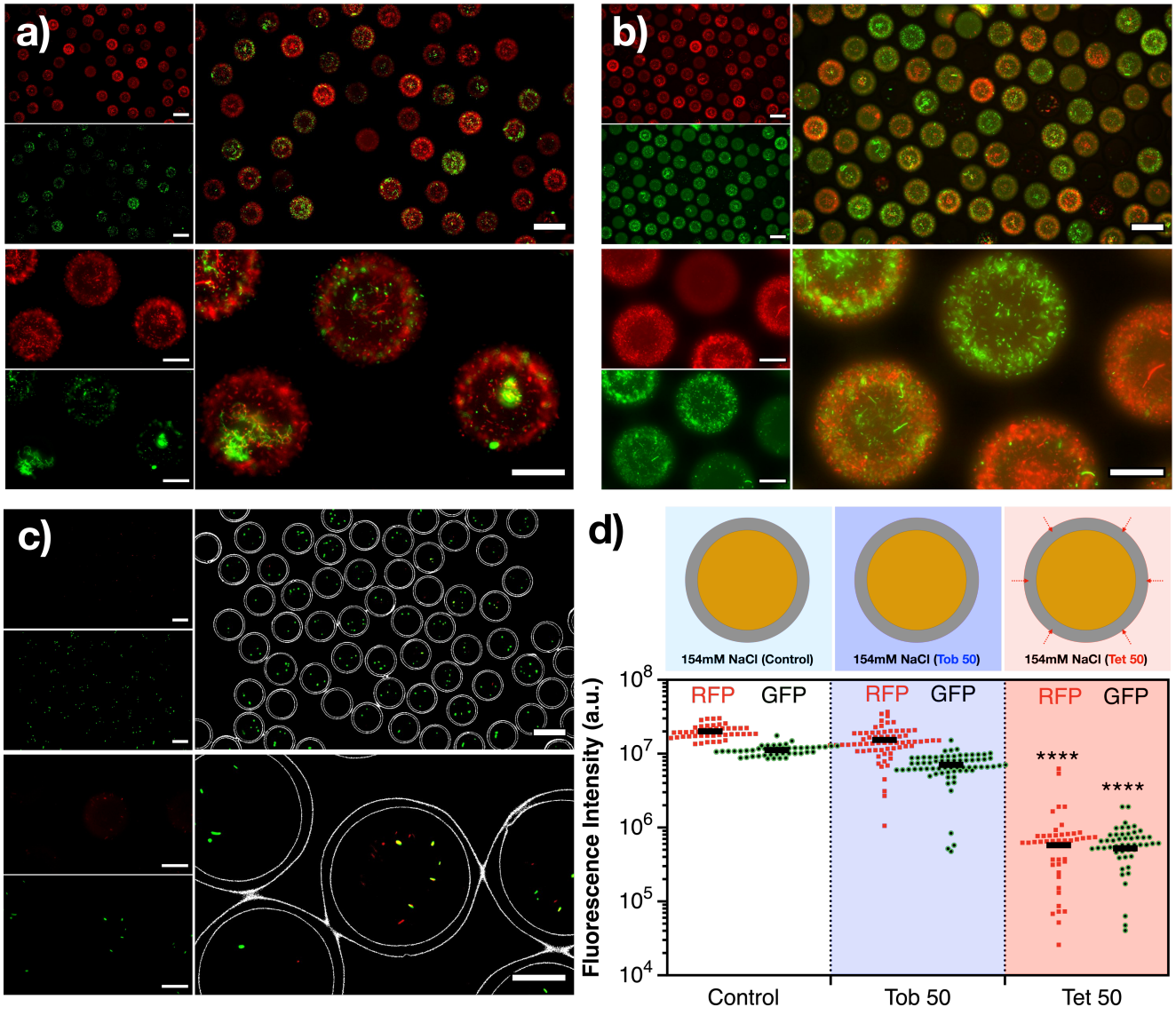

Figure 8. **(a)** Co-cultivation of GFP and RFP-expressing *E. coli* strains in PMDS nanocultures functionalized with Dimethylallylamine. **(b)** The exposure of the nanocultures to 50 µg/mL Tobramycin did not alter the growth dynamics of the cells. In the contrary, **(c)** exposure of the nanocultures to 50µg/mL Tetracycline led to the diffusion of the drug and the growth inhibition of both *E. coli* strains. **(d)** The fluorescence intensity was measure as a proxy for antibiotic diffusion in the functional membrane and cell viability. Reproduced with permission (Manimaran et al., 2020)

Recently, Usman et al. (2021) presented a novel microbial nanoculture system designed with a defined composition of PDMS-based hydride and vinyl with stable mechanical properties to house microorganisms in semipermeable nanocultures. The resultant polymer had lower mechanical strength as compared to the Sylgard 184. The brittle nature of the polymer provides enough mechanical stability to sustain a microbial microcapsule in various environmental condition and also facilitate the release of the microbes when subjected to mechanical stress lower than 1 MPa. The study has demonstrated that the tunability of microbial culture system can be achieved by varying the composition of vinyl and hydride introduced to build the polymer network in the presence of platinum catalyst.

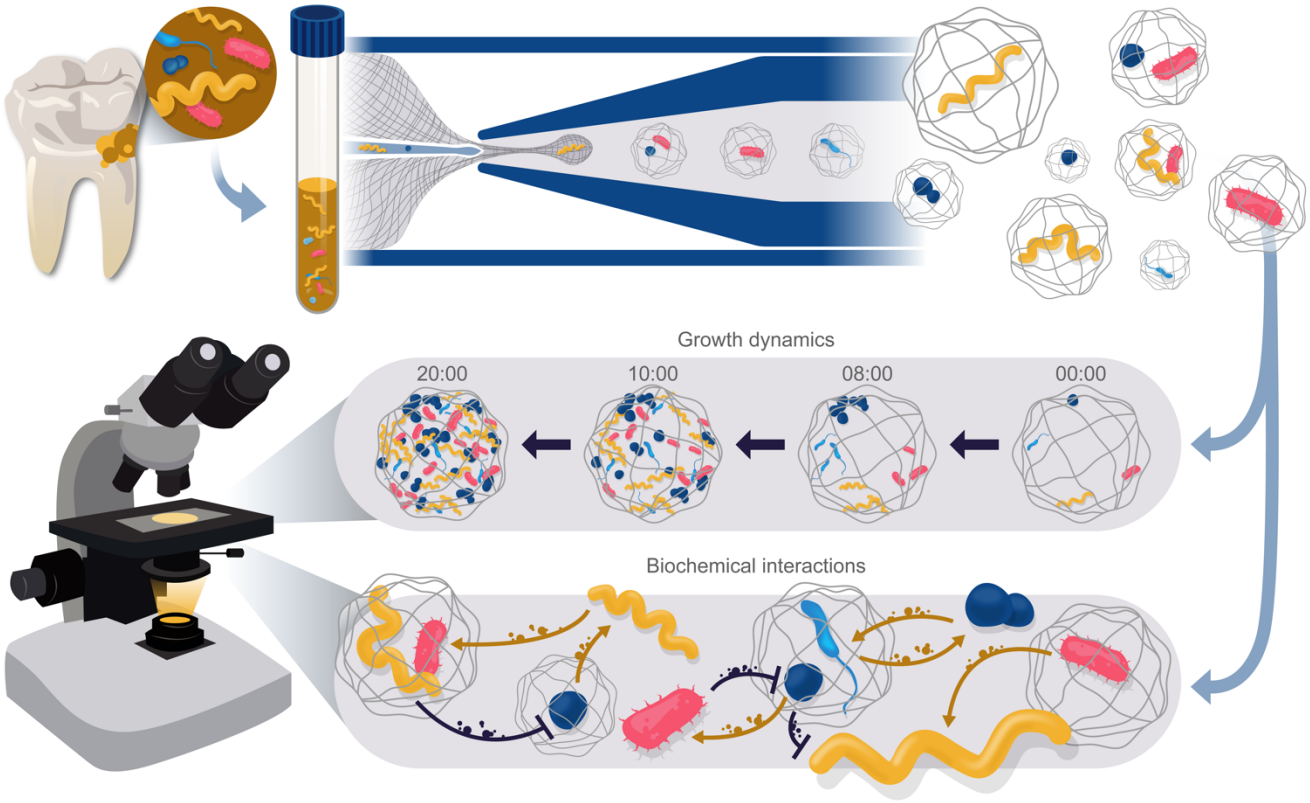

Figure 9. Flow chart for the assessment of oral microbial dynamics using droplet-based microsystems such as the nanocultures. The dental plaques are isolated from human subjects and stored into a saline solution. The aliquot is resuspended in culture broth at high dilution to grow the dental isolates into the nanocultures. Owing to their small volume, the growth dynamics of each encapsulated cell could be studied. The selective permeability of the nanocultures provide an extra advantage for facilitating the assessment of community dynamics based on chemical vs. physicochemical interactions.

Furthermore, the crosslinking density, the amount of free space within the polymer network, and the molecular selectivity of the membranes can be manipulated by introducing functional groups into the

shell of the nanocultures. Such approach lays the foundation for a unique platform to produce microsystems on demand, and to meet the growth requirement of the microbes being encapsulated. The development of such functionality would make possible the encapsulation of oral microbial species by chemically modifying the polymer network to become responsive to the physicochemical changes experienced by the oral environments and evaluate oral microbiome dynamics under various diet. For example, Manimaran et al. (2020) designed a low-pH responsive PDMS network with a *N,N*-Dimethylallylamine functionality to improve the permeability of the shell of the resulting nanoculture to tetracycline and control microbial growth (**Fig. 8**). The design of such smart semipermeable microniches, responding to microenvironmental changes, can help harness the diversity of entire oral microbiome, investigate and identify new oral species in a high throughput manner.

Thus, the encapsulation and compartmentalization of microbial communities into semi-permeable membrane such as PDMS can speed up the assessment of microbial interactions in various environmental conditions, especially for species typically found in low proportion. Because of their selective permeability, the nanocultures could serve to the bioprospecting of unculturable cells in the oral environments. Furthermore, the mechanical stability of the nanocultures can enable long-term study of microbial growth dynamics based on the diffusion of factors such as signaling molecules, antibiotics, and fluorescent labels capable of diffusing through the shell of the nanocultures (Niepa et al., 2016) (**Fig. 9**).

In conclusion, the microfluidics are a powerful technique for designing artificial microbial microniches and exploring the oral microbiome. they could help generate new assessment tools for the bioprospecting of the oral microbiome with an immense potential for elucidating oral microbial dysbiosis and pathophysiology. The nanocultures can help to monitor oral health by detecting oral pathogens as well as controlling the interactions between pathogenic and beneficial microbes. If further developed, such functional microsystems could facilitate the delivery of beneficial isolates into the oral cavity as oral probiotics to rebalance the oral microbiome and enhance oral health. The application of droplet-based microfluidics is an innovative approach that will aid in alleviating this substantial gap and in providing novel techniques in the treatment of oral diseases.

Acknowledgements

This work was supported by NSF Grant No. DMR-2104731 and through generous startup funds provided by the University of Pittsburgh. *Ekeoma U. Uzoukwu* was supported by the University of Pittsburgh's Swanson School of Engineering Pre-PhD scholars Program.

Competing Interests

The authors declare that there are no competing interests.

Author Contribution

All authors have reviewed and given approval to the final version of the manuscript.

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