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Current Opinion in
Endocrine and Metabolic Research

Review

Recent progress in analyzing the spatial structure of the human microbiome: Distinguishing biogeography and architecture in the oral and gut communities

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Abstract

Fueled by technological advances in methods for sample collection and preservation in sequencing studies and in advances in computational analyses of high-content image data, the spatial structure of the human microbiome is coming to light. In this mini-review, we summarize recent developments in our understanding of the structure of two human microbiomes: the lower gut and the oral cavity. We focus on only the most recent literature, and we make an important distinction between two forms of spatial structure governed by scale: biogeography and architecture. By segmenting the study of microbiome spatial structure into two categories, we demonstrate the potential to greatly advance our understanding of the mechanistic principles that link structure and function in the microbiome.

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Current Opinion in Endocrine and Metabolic Research 2021, **18:**275–283

This review comes from a themed issue on **Microbiome**

Edited by **Jethro Johnson** and **George Weinstock**

For complete overview of the section, please refer the article collection - Microbiome

Available online 26 April 2021

https://doi.org/10.1016/j.coemr.2021.04.005

Keywords

Oral microbiome, Gut microbiome, Microbiome biogeography, Microbiome architecture, Dental plaque.

Introduction

One of the most striking results to arise out of the study of the human microbiome has been the repeated observation of nonrandom structure in the community, whether it be across body sites or within host-associated biofilms [1–4]. Here, we refer to the macroscale structure of the microbiome, for example, on the order of mm to meters, as biogeography, akin to the distributions of flora and fauna at different geographic locations on earth [5]. The study of this level of structure comprises analyses of community composition and the ecological forces that drive these patterns [6]. We further identity a second level of structure in microbiome studies, that of the micron scale which we and others in the field have begun to refer to as architecture [7,8]. At this scale, the structures of human microbial communities retain characteristics of ecosystems, but especially in the case of biofilms, take on characteristics of metazoan tissues and organs, and therefore should be simultaneously considered from a cell biology perspective [9]. A number of comprehensive review articles have recently been published on this subject, to which we direct the reader [10–15]. Mostly for reasons of space limitations, here we restrict our in-depth discussion to the spatial structure of the microbiota of the lower gut and mouth, with a brief overview of the skin microbiome and provide a concise summary of the most recent literature within the framework of biogeography versus architecture.

Lower gut biogeography

Mostly through DNA analyses of stool, the bacterial component of the mammalian gut microbiome is known to be composed of thousands of phylotypes, predominantly in the bacteroidetes, firmicutes, actinobacteria, and proteobacteria phyla [1]. Comparative metagenomic analyses from different regions of the murine gut and between human ileostomy and colon biopsy samples have revealed a marked heterogenous distribution of these organisms along the length of the mammalian gut, hypothesized to be mediated primarily by host physiological variations [11]. The small intestine has a lower pH and a higher oxygen content than the colon and is influxed with digestive enzymes and surfactant in the form of bile acids [12]. Because most of the host's nutrient absorption occurs in the duodenum and jejunum, microbes there compete with the host for resources [12]. The host responds with higher antimicrobial concentrations in the small intestine than the colon. Thus, the community in the small intestine is

dominated by fast-growing, aerotolerant organisms that are capable of fermenting simple carbohydrates [16] and that tolerate low pH. Recent work has shown that the lumenal oxygen gradient from the proximal to distal gut is established and maintained in the absence of a microbiota in germ-free mice, mediated by lipid metabolism of the host endothelium, but that oxygen concentration further correlates with microbial bioload in conventional mice, suggesting a role for both the microbiota and host in oxygen depletion in the gut [17]. The shorter transit time of the fecal stream through the small intestine is hypothesized to contribute to the much lower bioload of organisms here than in the colon, which together with the cecum in, for example, the mouse, harbors both the highest biomass and the most microbial diversity in the gut [11]. In the cecum and colon, microbes including bacteroidacea and clostridia break down host-indigestible complex carbohydrates, in the slow-moving fecal stream [11]. A recent metagenomic analysis of biopsy samples from three sites, the terminal ileum, transverse colon, and rectum, from five subjects revealed that interindividual differences in community structure were larger than interintestine site differences and that all three biopsy communities were less diverse overall than feces, with higher relative abundance of bacteroidia in all three biopsy communities, a result that most likely reflects the difficulty in accessing the healthy human gut for this type of spatial analysis [18].

Nevertheless, the ever-decreasing cost of sequencing has allowed an explosion in comparative microbiome sequencing, and the observed differences in taxonomic composition along the lower digestive tract of laboratory mice and humans have been largely recapitulated in mammals, including wild mice [19] and tree squirrels [20], and correlations in community abundance differences and complex carbohydrate metabolizing capacity in the proximal versus distal gut of a freshwater gastropod [21].

Lower gut architecture

Microscopy applied to gut tissues prepared from animal models and human biopsies has revealed the striking nonrandom architecture of microbes in the colon, where there exists a near complete absence of microbes adjacent to the epithelium and the innermost dense mucus layer and an increasing abundance in the outer loose mucus and lumen, especially in regions of complex carbohydrate-rich, undigested food particles. The central importance of mucus in maintaining health and gut homeostasis has been well established through imaging [22], and specific microbial taxa that stimulate goblet cells to produce mucus, for example, *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii* [23], have been correlated with health, whereas epithelial-associated biofilms of *Bacteroides fragilis* [22] and the mucus-degrading species *Akkermansia muciniphila* [6] have been associated with inflammatory bowel disease. For imaging studies, tool development for preservation of the mucus layer during fixation and labeling has been a major area of research, and polymer-based chemistries have recently been demonstrated to well preserve host mucus and microbial cells in place [24,25].

With optimized protocols, Mark Welch et al. [26] used spectral imaging to map the architecture of a 15-member humanized community in the mouse colon (Table 1). Apart from the marked cross-sectional heterogeneity in total microbial distribution with regard to the mucus layer previously mentioned, no significant intertaxon spatial relationships were observed in the lumenal community, an observation that informed their hypothesis that the mixing forces present in the lumen are sufficient to distribute biochemical mediators both antagonistic and beneficial that might underlie structure in less dynamic environments. Using higher taxonomic level probes, Earle et al. [27] observed nonrandom distribution, for example, clustering of taxa in a mouse colon model, and further demonstrated that mice fed with a diet low in microbiota-accessible carbohydrates (MAC) resulted in a thinner host mucus layer and an increase in clustering of monophyletic bacteria, reinforcing the idea that diet and mucus integrity are interrelated, mediated by the gut microbiota in an architecturally nonrandom manner (Table 1 & Figure 1a).

Recently, metagenomic sequencing of encapsulated microbial consortia has been applied to the murine gut. The MaPS-seq approach of Sheth et al. [28] circumvents the problems associated with mucus and tissue preservation as well as the limited breadth of probe-based imaging methodologies and has corroborated both previously described biogeographical variations and clustering of phylogenetically related organisms in response to perturbations in diet (Table 1). This tool will undoubtedly serve as an important complement to ongoing imaging studies aimed at elucidating the roles of intermicrobial cell adhesion; biochemical gradients; scaffolds provided by undigested food, shed epithelial cells, and mucus; and local immune responses in structuring the gut community [11]. As well, studies of architectural dynamics in the gut microbiome require live cell, time-lapse imaging, and the optical transparency of zebrafish larvae makes this organism an exquisitely well-suited model for addressing these types of questions [29]. Recently, the role of microbial motility, mediated by flagellar-based chemotaxis, in a proinflammatory *Vibrio* sp. was identified as a potential contributor to maintenance of this taxon in the face of peristalsis and unidirectional flow in the zebrafish gut lumen, with implications for human health as several gut pathogens including *Salmonella enterica* and *Escherichia coli* possess flagellar motility [30].

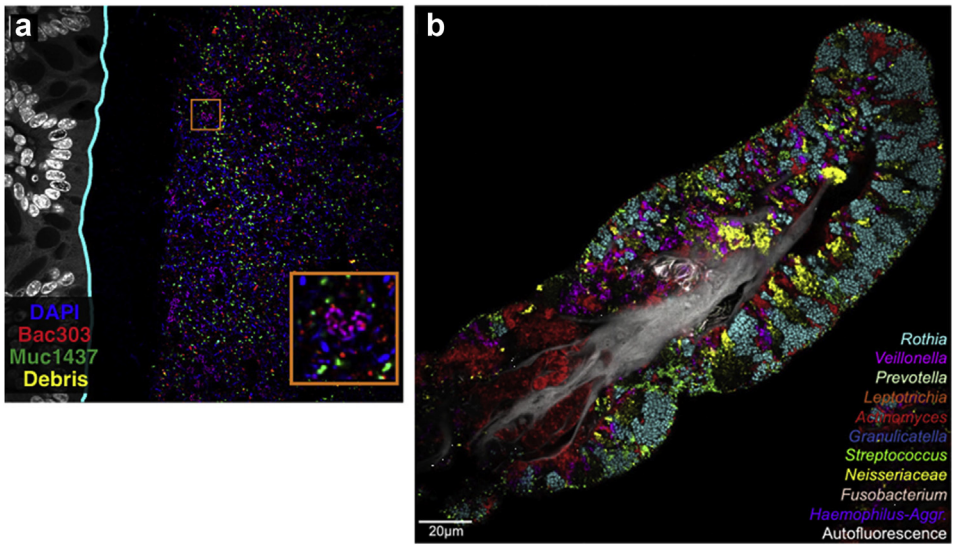
Table 1

Established and emerging technologies for mapping the micron-scale architecture of the human microbiome.

Technology	Pros and cons	Recent examples of applications to the human microbiome
Fluorescent immunohistochemistry (FI-IHC)	Pros: Can identify specific strains of microbes. Can be used in combination with FISH labeling to identify specific host cells, esp. immune cells. Cons: Requires cultivation of microbes along with generation and validation of antibodies. Limited multiplex capacity, although serial immune labeling strategies can be used.	Strain level identification of *Streptococci* in dental plaque biofilms combined with FISH [73]. Spatial analysis of *Helicobacter pylori* and mitotic epithelial cells in the murine stomach [27].
Fluorescence *in situ* hybridization (FISH)	Pros: Can identify microbial taxa without the need to culture them. Cons: Multiplex capabilities are limited by fluorophore cross talk. Requires databases of full-length 16S sequences for probe design. Probes may cross-hybridize to 5S or 23S sequences of off-target organisms.	Spatial analysis of intact supra and subgingival dental plaque biofilms on extracted teeth [54].
Imaging fluorescent protein–expressing bacteria	Pros: Can image live microbial cells and dynamic interactions. Cons: Requires engineering genetically tractable strains to express fluorescent proteins.	Spatial and temporal mapping of colonization, growth, and motility model microbiomes in zebrafish larvae [74,29].
Combinatorial labeling and spectral imaging FISH (CLASI-FISH)	Pros: Can identify microbial taxa without the need to culture them. Multiplex capacity has been extended to 16 or more targets. Cons: Combinatorial label identification can be ambiguous when microbial cells are in close contact. Probe design and validation is low throughput and highly laborious. 16S targeting may only provide genus level or higher taxonomic resolution.	Spatial mapping of up to 15 taxa in extracted dental plaque, humanized mouse colon, and human tongue biofilms [75,50,26,58].
High-phylogenetic-resolution microbiome mapping by fluorescence *in situ* hybridization (HiPR FISH)	Pros: Can identify microbial taxa without the need to culture them. Multiplex capacity has been extended to tens to hundreds of targets. Probe design is high throughput, and probes can discriminate closely related organisms. Cons: As for CLASI-FISH, combinatorial label identification can be ambiguous when microbial cells are in close contact within the axial resolution limit of the microscope.	Highly quantitative spatial and colocalization analyses of up to 20 taxa in human dental plaque and murine colon tissues [59].
Raman microspectroscopy	Pros: Allows label-free imaging of targets with light microscope resolution. Cons: Requires libraries of known molecular signatures for taxonomic identification.	Label-free spatial analysis of a 5-species subgingival plaque biofilm model [76].
Nanoscale secondary ion mass spectrometry (Nano SIMS)	Pros: Can measure metabolic fluxes in single microbial cells. Can be combined with FISH for correlative single-cell taxonomic identification. Cons: Destructive to the sample. Requires doping of the sample with radioisotopes and specialized instrumentation.	Spatial analysis of *in vivo* growth rates of *Clostridium* cells in a gnotobiotic mouse GI tract [77].
Metagenomic sequencing of encapsulated microbial consortia (MaPS-seq)	Pros: Can provide whole microbial genome sequence information with micron-scale resolution on consortia of microbes. Provides information on cell–cell interactions. Cons: Requires encapsulation and disruption of community architecture. Resolution is limited to consortia of microbes.	Spatially resolved microbial consortium analysis in the murine intestine and identification of diet-driven clustering patterns [28].

Technologies are presented here along with brief descriptions of their pros and cons. Some recent applications of each technology are listed as examples and are not meant to comprise a comprehensive list of all applications of each technology to the human microbiome.
GI, gastrointestinal.

Figure 1



Architectural features of the microbiome are governed both by ecological and cell biological principles. (**a**). A FISH-labeled image of a murine gut community in an animal fed with a low MAC diet. DAPI labels all bacteria, Bac303 labels bacteroidales, and Muc1437 labels *Akkermansia*. Compared with control animals, low MAC condition microbiota are located closer to the host epithelium (cyan mark) because of reduced mucus thickness. Monophyletic clusters of microbes are present (inset), suggesting functional interaction mediated by close spatial proximity in the face of lumenal mixing forces. Reprinted with permission from Earle et al., [27] 2015, Cell Host & Microbe. (**b**). A FISH-labeled image of a human tongue dorsum community on a shed epithelial cell. Probes for 10 abundant and prevalent taxa in the human tongue dorsum community show patchy distributions, nucleated around a shed host epithelial cell. The architectural arrangements of the different taxa are hypothesized to result from population founder effects and subsequent growth dynamics as well as intertaxon physical and chemical interactions. Reprinted with permission from Wilbert et al., [58] 2020, Cell Host & Microbe.

One stark contrast to the exclusion of microbes from the epithelia by mucus has been the long-standing observation of members of the segmented filamentous bacterial cluster of microbes, closely associated with Peyer's patches in the mammalian gut; this observation is hypothesized to reflect the role of these organisms in maintaining inflammatory homeostasis via direct association with immune cells [31]. The mammalian gut is highly innervated, and recently, nociceptor neurons were identified to mediate protection against *Salmonella* infection through downregulation of M cells in Peyer's patches to allow expansion of segmented filamentous bacterial cells, an example of the important role of spatial structure in the interplay between the host immune system and commensal microbiota in mediating colonization resistance to exogenous microbes [32,33]. The binary cross talk between gut microbiota and the host is further exemplified by the recent observation of correlations in protein turnover in epithelial cells and mucus to spatial location in the gut and the microbiota [34] and observations of increased B cell accumulation and clonal expansion from the cecum to the sigmoid colon, correlated with increased immune-reactive bacterial species [35].

Specific microbial taxa, including the conspicuous oral commensal *Fusobacterium nucleatum*, have been observed in direct association with colonic tumors, while significantly reduced in abundance in nearby non-neoplastic tissue [36,37]. The role of microbes in driving tumor progression versus merely localizing to a tumor-derived favorable environment is beginning to be teased apart [38]. Biofilms which comprised *E. coli* and *B. fragilis* were observed in proximity to benign precursor lesions (polyps) in biopsy specimens from patients with a history of familial adenomatous polyposis. Dejea et al. [39] demonstrated that the two bacterial species could induce tumor progression and increased mortality when inoculated into tumor-prone mice, strongly suggesting a synergistic mechanism by which these microbial pathogens contribute to tumor progression. In summary, many of the previously observed microbiome-associated health and disease processes in the mammalian gut are beginning to be better understood from a spatial viewpoint.

Oral cavity biogeography

Like the gut, the bacterial component of the oral microbiome as a whole is comprised mostly of members of the firmicutes, proteobacteria, actinobacteria, and bacteroidetes phyla with the additional orally abundant fusobacteria and spirochaetes [40]. The mouth comprises a multitude of substrates for microbial colonization including teeth, tongue, buccal mucosa, and gingiva. Early culture-based studies revealed that the different oral sites supported

different communities, and this early work has been confirmed with modern molecular-based metagenomic sequencing [41]. In stark contrast to the gut, the different habitats within the human mouth are easily sampled, for example, the initial Human Microbiome Project analyzed nine different oral communities: saliva, buccal mucosa, gingiva, palate, tonsils, throat, tongue and teeth, and both supra and subgingival plaque [1]. The biogeography of the oral microbiome distributed on these different surfaces is hypothesized to reflect the different chemistry, topography, and stability of the substrates and their immediate environment [15]. The soft tissues of the oral cavity, like the gut epithelium, are constantly shed and therefore select for fast-growing organisms that comprise minimally diverse and low-bioload communities.

Unique in the oral cavity is the body's only permanent tissue, the teeth. This substrate supports the growth of extraordinarily diverse communities that may develop via ecological succession over long time periods, in humans especially in the absence of adequate oral hygiene or in tooth aspects protected from regular dental plaque removal, into extraordinarily diverse climax communities [42]. Adhesion of microbes to the saliva-coated enamel and to each other is a major characteristic of supragingival species because without it, the shear forces of saliva experienced across the dentition and subsequent swallowing would remove nonadhered cells [43]. The localized accumulation of waste products from fermentative acidogenic organisms is known to mediate the localized demineralization of tooth enamel in the pathogenesis of dental caries, in the face of the pH buffering saliva [13]. The observation that across populations, dental caries occur in a nonrandom fashion with regard to tooth number and aspect informed the hypothesis that the composition and especially the flow rate of saliva over the tooth surface contribute to the community composition on teeth. Proctor et al. [44] tested this hypothesis by rigorously sampling and sequencing extracted DNA from dental plaque communities from different teeth and soft tissues distributed within the mouth. The authors identified a proximal-to-distal gradient in community composition among low-abundance taxa for both hard and soft tissue–associated communities. This gradient was not seen in patients with Sjögren's syndrome, an autoimmune disorder associated with severely impaired salivary gland function, which suggests that the proximity to salivary glands may mediate this community gradient in non-Sjögren's volunteers. Especially relevant to our discussion of biogeography, Proctor et al. further assayed the manner of dispersal within the mouth by fitting their data to two different models: island hopping or Euclidean distance from glands. Their data did not support the island-hopping model, in which species can migrate only stepwise from one tooth to an adjacent one.

In contrast to the highly oxygenated, saliva-bathed supragingival surface of the teeth, the environment of the subgingival pocket is devoid of oxygen, and the microbes there are bathed in gingivocrevicular fluid, composed mostly of serum exuded from the gingival epithelium [45]. As such, the subgingival dental plaque community is enriched in strict anaerobes and slow-growing, asaccharolytic organisms that use serum-derived amino acids as their primary carbon source [13]. Increases in subgingival bioload and changes in community composition commensurate with host immune system engagement are known to mediate the transition from health to periodontal disease [46,47], and metatranscriptomic analyses of early stages of periodontal disease are providing insight into the initiation of this chronic, progressive disease [48].

Recent deep sequencing approaches have revealed that for the most part, the microbiomes that assemble at different oral habitats are related, that is, they are composed of the same genera, but have marked species specificity [49]. This observation has led Mark Welch et al. [50] to propose their "site-specialist hypothesis" of oral microbial biogeography, which states that different sites in the oral cavity support different communities at the species and strain level because microbes have adapted to one another and coevolved with the host.

Oral cavity architecture

Analysis of early electron and light micrographs of dental plaque labeled with nonspecific stains revealed striking nonrandom architectures, mediated by patterned development of morphologically similar and dissimilar cells in direct contact and projecting tens of microns perpendicularly to the saliva-coated tooth surface [51–53]. Combined with longitudinal culture-based analyses, these observations identified the importance of ecological succession in the formation of dental plaque. The recent application of fluorescence *in situ* hybridization (FISH) and confocal microscopy to the study of intact oral biofilms has begun to allow the taxonomic identification of the organisms in these highly patterned structures (Table 1).

Multispectral imaging combined with FISH on extracted supragingival dental plaque revealed striking "hedgehog" structures, which comprised filamentous organisms of the genera *Corynebacterium*, *Fusobacterium*, *Leptotrichia*, *and Capnocytophaga* [9]. The *Corynebacterium* filaments were observed in close association with cells of the genera *Streptococcus and Actinomyces* at their base and were decorated at their distal tips with various cocci of the genera *Streptococcus*, *Haemophilus or Aggregatibacter*, *and Porphyromonas*. These so-called corn-cob structures had previously been described without taxonomic resolution, and morphologically similar corn-cobs have been identified in other plaque samples with hyphal

Candida albicans as the filament, decorated with *Streptococci* [54,55].

In teeth extracted from toddlers with severe early childhood caries, FISH labeling revealed direct associations between *C. albicans* and nonmutans *Streptococci* and diffuse associations between *C. albicans* and *Streptococcus mutans*, along with the presence of *S. mutans*–derived exo-glucosyltransferases (Gtf)s on the surface of *C. albicans* hyphae, an observation with clinical relevance as the production of exopolysaccharides through Gtfs is hypothesized to mediate sequestering of pH reducing lactic acid from *S. mutans* [4,54,56]. In the same samples, dome-like structures of *S. mutans* surrounded by nonmutans *Streptococci* were observed. The significance of these structures in caries pathology is yet to be identified.

Observations of dental plaque architecture are beginning to be assimilated with metagenomic and metatranscriptomic data to inform hypotheses that correlate structure with function in these communities. The patterned development of the supragingival community is hypothesized to form through growth and differential gene expression in response to external environmental cues and forces mediated by the host and the microbial cells themselves. This dynamic process, mediated by both extrinsic (host) and intrinsic (microbe) forces, is hypothesized to generate functional niches within these cellular structures [9]. Intriguingly, recent live cell spectral imaging studies of *in vitro* communities revealed the capacity of *Capnocytophaga* filaments to transport other species along their surfaces, providing a potential mechanism to introduce nonmotile species into their preferred niches during dynamic biofilm maturation [57].

In a recent spectral imaging study of epithelial cells collected from the tongue dorsa from volunteers, Wilbert, et al. [58] described multispecies biofilms that decorate the host cells. In contrast to supragingival dental plaque, these biofilms were observed to be less diverse at the genus level and were comprising a high number of fast-growing fermentative organisms, which might reflect the nature of the biofilm scaffold: here an epithelial cell destined to be shed and swallowed by the host rather than the permanent surface of the dentition (Figure 1b). Nonetheless, striking patterns in biofilm architecture were observed with different taxa arranged in patches, stripes, and regular geometries, for example, cones, which could be interpreted, at least in some part, as snapshots of time-dependent growth patterns in response to variable host nutrient intake in the form of fermentable carbon sources.

As noted previously regarding gut architecture and imaging, the design, synthesis, and validation of FISH probes can be a bottleneck in achieving true system level imaging of the oral microbiome. Especially relevant to this environment where direct cell-to-cell contacts are known to be important, the recent development of high-throughput probe design, FISH labeling, and error-free image analysis via high-phylogenetic-resolution FISH has been validated with longitudinally collected, extracted plaque samples, and its future application to intact biofilms will allow analysis of biofilm architecture with unprecedented taxonomic breadth (Table 1) [59].

Spatial structure of other human microbiomes

The biogeographies and in some cases architectures of other human microbiome communities are beginning to be elucidated through metagenomics and imaging in tissue samples and animal models inclusive of the communities associated with the urogenital tract, upper and lower respiratory tract, and others [60–64]. Especially, the biogeography of the skin has recently been well described, first with 16S amplicon sequencing and more recently with metagenomics, to identify the spatial distribution of the bacterial, fungal, and viral members of the skin microbiome [2,65–67]. Sampling of up to 20 different skin sites has consistently revealed that the biogeography of the skin microbiome is driven by the environmental characteristics of the body at that site, for example, whether the skin at that site is moist, dry, or sebaceous [65,67]. Correlative 16S amplicon sequencing and mass spectrometry have been applied to the skin to reveal that molecular composition of the skin has a distinct biogeography mediated by both host cells, microbes, and the application of hygiene products [3]. Intriguingly, the use of hygiene products at specific body sites, specifically underarm deodorant and foot powders, has recently been correlated with increased microbial diversity [68].

The micron-scale architecture of the skin microbiome is yet to be directly observed with taxonomic resolution; however, indirect evidence for direct physical association or close proximity between specific skin commensal bacteria and cells of the host immune system has been reported. Scharschmidt et al. [69] demonstrated that regulatory T cells are recruited to developing hair follicles in postnatal developing mice in a commensal microbiota-dependent manner. Linehan et al. [71] and Naik et al. [70] have observed that *Staphylococcus epidermidis* can trigger expansion of antigen-specific cytotoxic T cells but that this upregulation is mediated by dendritic cells, which suggests a need for direct physical contact between commensal microbes and certain host immune cells in establishing proper immune system development and downstream inflammatory responses to pathogens. Direct observation of skin microbial architecture and its clinical importance has recently been described in a chronic skin wound

model, in which the direct physical contact between a skin commensal, *Citrobacter freundii*, and the opportunistic pathogen, *C. albicans*, was observed to induce the yeast-to-hyphae transition in *C. albicans*, resulting in increased neutrophil death [72]. In conclusion, the direct observation of microbiome architecture and physical interactions with the host has been limited outside of the oral cavity and gastrointestinal tract. We posit that the direct observation of skin and other microbiome structures and interactions between microbes and host cells may be hampered by the dynamic and transient nature of these interactions. The continuous shedding of commensal-associated epithelial cells is in direct contrast to the permanent, nonshedding human tooth surface which may have favored the evolution of stable, highly complex biofilms on the teeth.

Conclusion

In this mini-review, we have highlighted recent progress in studying the spatial structure of two human microbiomes: the lower gut and oral cavity. As our observations of the microbial community structure grow, we have highlighted examples where our understanding of some of the well-described correlations between microbiome composition and host health and disease state is improved with concomitant information from the spatial domain.

We further make an important distinction between two levels of scale in spatial analyses of the microbiome: biogeography and architecture. Descriptions of spatial structure at any scale, whether they be qualitative or quantitative, are only a form of observation. To achieve a mechanistic understanding of how the microbiome functions in promoting health or allowing disease through the use of different omics technologies and their application to controlled tests of hypotheses in, for example, animal models, it will be necessary to consider not just microbial community spatial structure, but the different fundamental principles that shape structure at different scales. The ecological principles such as population dispersal and abiotic or host-mediated environmental gradients that govern community structure and function at the macroscale are fundamentally different from the cell biological principles such as cell−cell adhesion and molecular-mediated transport that act at the microscale. With this framework, the multitude of structure−function relationships within the microbiome and between microbiome and the host isare being uncovered.

Conflict of interest statement

Nothing declared.

Acknowledgements

This work was supported by U.S. National Institutes of Health grant DE028042 to A.M.V.

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