The microbiome – the explanation for (almost) everything?

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Introduction

The human microbiome — the collection of microorganisms that inhabit our bodies — has been suggested to play a role in a vast number of diseases, as well as in the maintenance of normal health. There has been an exponential rise in such reports, in part because of new rapid and affordable sequencing technologies. However, developing our understanding from qualitative description to quantitative modelling is essential for incorporation into routine clinical care. Here, we cover the basics of microbiome research and discuss some important examples relevant for paediatric infectious diseases.

The birth of a child is a transition to the external world, bringing with it a sudden exposure to microorganisms. Their previously sterile body is rapidly colonised, with the initial exposure to vaginal flora followed by environmental bacteria. At first these initial colonisers may be random, but within months they establish and develop into stable microbial communities. These communities can remain throughout a child's life; collectively, they form the human microbiome.

This description of birth underlines our changing understanding of human-microbe relationships. In traditional paradigms of infectious disease, bacteria colonising a young child instinctively suggests the need for urgent intervention. The realisation that the establishment of a stable microbiome is necessary for proper immune development, resistance to further colonisation by more pathogenic bacteria, and potentially much more, has led to a shift in the perception of the role of bacteria in health and disease. However, the considerable hype around the microbiome has also engendered scepticism. In this review, we consider the relevance of the human microbiome for paediatric infectious disease. We focus only on bacteria (although generally the microbiome encompasses all microorganisms including viruses, archaea, and fungi) and use the term 'microbiome' interchangeably with 'microbiota'.

Good bacteria, bad bacteria

Popular depictions of the microbiome suggest looking after 'good' bacteria while looking out for the 'bad'. Does this mean the infectious disease specialist must now balance nurturing a patient's healthy microbiome alongside their other responsibilities of treating potentially pathogenic infections? Unfortunately, we do not currently know what the 'healthy microbiome' looks like, or if such a concept even meaningfully exists. We all have our own unique microbiome. Even within each of us there is no single microbiome. Each niche within the body harbours its own populations of bacteria — the same body sites from different individuals show more similarities than different body sites from the same individual — and although exchange between these populations occurs, they remain distinct. For example, the oral habitat contains different microenvironments with correspondingly different microbial communities (1) and the gut microbiome varies along the length of the gastrointestinal tract.

Generally, the distinction between 'pathogen' and 'commensal' appears not to be a hard-and-fast one. Many species are normal members of the gut microbiome, carried by many healthy individuals, but in some circumstances can cause life-threatening infection. These circumstances are often closely connected to the state of the microbiome. *Clostridium*

difficile infection (CDI) is a particularly interesting example for the infectious disease specialist, and demonstrates how the microbiome can contribute to treatment. Individuals who suffer from CDI have typically recently undergone broad-spectrum antibiotic treatment, which reduces the microbiome's species diversity. The opportunistic overgrowth by *C. difficile* is the manifestation of the deeper community-wide dysbiosis; it is the symptom, not the cause. The success of conventional treatments with antibiotics for *C. difficile* is variable and relapse is common. An effective treatment is faecal microbiota transplant (FMT): stool from a healthy donor is transplanted into the sufferer, suppressing the overgrowth and restoring a healthy gut microbiome. Consistent success rates of above 90% have been reported in multiple trials (2). Although the mechanism by which FMT actually works is still not entirely understood, it somehow restores the gut microbiome to a community state that controls the growth of *C. difficile*. Indication that the general principle of 'dysbiosis' is important — rather than specifically *C. difficile* — comes from the observation that intestinal colonization can also occur from other parts of the human microbiome during gut dysbiosis e.g. oral *Klebsiella* species can ectopically colonize the intestine under certain conditions (3).

From medium, to markers, to metagenomics

Analysing the microbiome's role in disease has historically been challenging due to the difficulty of culturing bacteria i.e. finding the specific set of medium and conditions that will favour the growth of an organism adapted to life within the human body. Technological developments in sequencing and associated decreases in costs have allowed the 'culture-free' characterisation of clinical samples, facilitating a dramatic expansion of microbiome research. However, the resulting larger quantities of data present correspondingly larger opportunities for substandard analysis. It is important to understand the underlying technologies in order to avoid being dazzled by novelty. Here, we briefly cover two important sequencing approaches.

The taxonomic composition of a sample is often quantified with 16S ribosomal RNA (rRNA) gene sequencing. The 16S rRNA gene is around 1,550 base pairs long and is universal among bacteria, because it codes for an important part of the ribosome: the molecular machine that translates and assembles proteins from messenger RNA (mRNA). As translating proteins is a fundamental cellular process, certain regions of the 16S rRNA gene are under high selection to preserve ribosomal function, and are highly conserved. However, the gene also codes for regions that loop around within the ribosome's structure without directly contributing to function. These regions are not under high selection and so tend to accumulate more mutations, making them more variable and meaning that sequences can be associated with bacterial taxonomy. This combination of variable and conserved regions means that universal DNA primers can be designed to target a conserved region, facilitating replication along the sequence of DNA which continues into a hypervariable region. When applied to a sample containing many different taxa, relative abundances can then be calculated by grouping similar sequences into units, often called Operational Taxonomic Units (OTUs) or phylotypes.

The apparent sophistication and ease of 16S rRNA gene sequencing compared to traditional clinical microbiology can disguise subtleties in analysis and data interpretation. Bacteria can carry multiple copies of the gene, primer bias can result in preferential amplification of certain taxa, and different regions can detect phyla in different proportions, thus biasing diversity metrics. Furthermore, relative abundances do not necessarily correspond to true numbers of bacterial cells (although differential abundances across samples for a particular taxa can still be calculated). Importantly, the 16S rRNA gene contains only a small fraction of the total genetic diversity in the microbiome and no direct information on function. It may

not be sufficient for answering questions of clinical significance, particularly if these concern function rather than taxonomy.

Contrastingly, in shotgun metagenomic sequencing all DNA in a sample is extracted and sequenced. While this brings with it considerable advantages — increased taxonomic resolution and functional characterization — the analysis is even more challenging than for marker gene data. It should be stressed that while metagenomic sequencing is popularly presented as intrinsically superior to 16S rRNA gene sequencing, it would be a mistake to assume that a study using metagenomics is necessarily better. The research question, study design, sample size, and cost are all important factors when choosing an appropriate technology e.g. despite the hype around next-generation sequencing, if testing for a known organism it can be possible to deliver a much stronger conclusion for a fraction of the cost with a well-designed PCR.

A cursory search of PubMed for a disease of your choice will almost certainly reveal putative associations with the microbiome. Unfortunately, many microbiome studies represent opportunistic 'data-fishing' expeditions with small sample sizes and confounded variables, so their conclusions should be taken with an appropriate pinch of statistical salt. Notwithstanding such a 'microbiome of the gaps' approach to unexplained variation in disease, good microbiome studies do exist. In the following section, we discuss some well-supported findings.

What shapes the microbiome?

The early-life establishment of the microbiome appears to be driven largely by environmental factors rather than the genetic relatedness of individuals. One study used salivary samples from a large extended family to show that shared household had the biggest effect on salivary microbiome composition (4). Here, the taxonomic resolution provided by 16S rRNA gene sequencing was sufficient for the research question, and the study design incorporating human genetics, household information, and other variables allowed for the simultaneous analysis of their effects on composition. Other studies have shown similar results for the gut microbiome, raising the intriguing possibility that the microbiome could play a role in early life and the development of paediatric diseases that show familial aggregation such as inflammatory bowel disease (IBD). Even before birth, the maternal vaginal microbiome has been suggested as a possible source for placental infection, with the presence of specific bacteria in placental tissue associated with a smaller newborn (5). On the one hand, evidence that the environmentally-determined microbiome can influence the development of disease is exciting, as it suggests that the microbiome is not determined by our genetics and may be modulated to treat disease. However, if these environmental effects are indeed so dominant, it may prove difficult to overcome them.

A major factor that shapes the microbiome is antibiotic use. Antibiotic use in early life has been highlighted as a particular concern, with the provocative suggestion that it may cause an increased incidence of autoimmune conditions due to inference with normal immune development. While convincing long-term cohort studies incorporating sequencing data are yet to be carried out, microbiome studies have already provided interesting insight into this crucial phase of microbiome development. Yassour et al. used metagenomic sequencing of monthly stool samples from 39 children during the first three years of life to show conclusively that antibiotic use was associated with a gut microbiome composed of fewer species (i.e. less diverse) (6). The combination of metagenomic data with clinical prescribing information allowed them to demonstrate that antibiotic resistance genes carried on transferable genetic elements increased in abundance immediately after antibiotic treatment and persisted after its cessation. These findings support concerns over the long-term risks of

antibiotic use, both in the development of resistance but also in terms of possible detrimental effects on the individual's health due to decreased diversity.

Diversity and normality

The overgrowth of *C. difficile* after antibiotic treament is not the only association between microbiome diversity and health. For example, children undergoing haematopoetic stem cell transplant (HSCT) for underlying immune conditions are typically immunocompromised or immunosuppressed before transplant, meaning they are placed on high levels of antibiotic prophylaxis. Low gut microbiome diversity at the time of engraftment has been significantly associated with lower survival in a multivariate model taking other clinical variables into account in adults (7), possibly connected to an increased risk of graft-versus-host-disease (GVHD). In children, Simms-Waldrip et al. observed that anti-inflammatory *Clostridia* species were depleted in GVHD in a small sample of paediatric HSCT patients, suggesting the depletion of normal gut microbiota by antibiotic prophylaxis — specifically, clindamycin — as the driver (8). Interestingly, in cancer treatment immune checkpoint inhibitors used to treat tumors fail to work in some individuals due to abnormal gut microbiome composition; the efficacy of these inhibitors can be restored by probiotics (9).

Insert Figure 1 here

Figure 1: Conceptual pictures of the microbiome can be converted into mathematical models to test hypotheses. (a) A schematic concept of a 'landscape' of possible microbiome states defined by their species diversity. Initially, the microbiome is at healthy equilibrium. A perturbation (e.g. by antibiotics) can then lead either to a return to equilibrium ('Return', green arrow), or to a transition to an alternative state ('Transition', red arrow). (b) Simple mathematical models based on these two possibilities can then be developed. (c) These models can be fitted to real data (e.g. a week-long course of clindamycin) and their fit compared, allowing a test of the hypothesis that the microbiome has transitioned to a different diversity state. For further details, see Shaw et al. (10).

The feasibility of using probiotics to maintain a healthy or 'normal' gut microbiome in a general setting is not yet established. Part of the difficulty is the lack of clarity about what exactly constitutes 'normal'. It is clear that abnormal disturbances to the microbiome like antibiotics do increase risks in specific clinical settings, but are these generalizable? It has been established that even short courses of antibiotics can lead to long-term depletions in gut microbiome diversity, and even alternative states of the microbiome, but whether these represent clinically significant departures from normality remains a matter of debate. We believe there is a role for modelling efforts to contribute to our understanding of the dynamics of microbiome reconstitution (10) (Figure 1). Understanding how to start the quantification of the general health impacts of antibiotic use could inform more appropriate prescribing. Current practices are largely empirical, applying a precautionary principle both to the use of antibiotics and to the duration of courses. The likelihood of negative impacts on the patient's microbiome should be taken seriously when weighing up the costs and benefits

of antibiotics. Improved diagnostics, possibly informed by sequencing approaches, could contribute considerably to this area.

Conclusion

It is now clear that the microbiome should not be overlooked. This is particularly true when considering paediatric infectious diseases, which may be manifestations of deeper problems during microbiome and immune development. New tools for quantifying the microbiome offer great promise for incorporating ‘microbiome thinking’ into routine clinical care. However, the microbiome cannot (and should not) be the explanation for every unexplained problem. It is a clinical factor which can be either relevant or irrelevant depending on the circumstances. If the microbiome is to be incorporated into future treatment approaches, a critical appraisal of the evidence, guided by an understanding of the technologies which underpin our knowledge, is essential.

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