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Commensal Koch's postulates: establishing causation in human microbiota research

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Advances in high-throughput sequencing technologies and the development of sophisticated bioinformatics analysis methods, algorithms, and pipelines to handle the large amounts of data generated have driven the field of human microbiome research forward. This specialist knowledge has been crucial to thoroughly mine the human gut microbiota, particularly in the absence of methods for the routine cultivation of most enteric microorganisms. In recent years, however, significant efforts have been made to address the 'great plate count anomaly' and to overcome the barriers to cultivation of the fastidious and mostly strictly anaerobic bacteria that reside in the human gut. As a result, many new species have been discovered, characterised, genome sequenced, and deposited in culture collections. These continually expanding resources enable experimental investigation of the human gut microbiota, validation of hypotheses made with sequence-based analyses, and phenotypic characterisation of its constituent microbes. Herein we propose a variant of Koch's postulates, aimed at providing a framework to establish causation in microbiome studies, with a particular focus on demonstrating the health-promoting role of the commensal gut microbiota.

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Box 1 Postulates for defining health-promoting microorganisms commensal to humans.

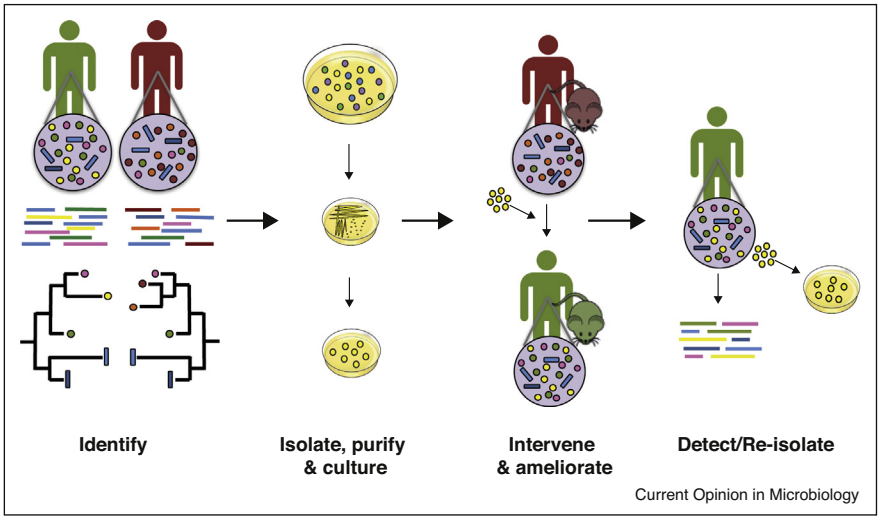
1: The commensal strain is associated with host health and is regularly identified in healthy hosts, but less frequently in diseased hosts.
2: The commensal strain can be isolated as a pure culture and grown in the laboratory.
3: The commensal strain ameliorates or mitigates disease when introduced into a new host.
4: The commensal strain can be detected following its introduction into a host to which health was restored.

Original Koch's postulates
1. The microorganism must be present in every case of the disease.
2. The microorganism must be isolated from the diseased host and grown as a pure culture in the laboratory.
3. The microorganism must cause the same disease when introduced into a new host.
4. The microorganism should be recovered from the new host.

Historically, microbiologists focused on the isolation and characterisation of food spoilage and disease causing microorganisms, due to the inherent socio-economic and health impacts that motivated their investigation. In 1884, Robert Koch's investigations on tuberculosis led him to propose the concepts that formed the basis of his famous postulates (Box 1) [1,2]. For over 100 years these fundamental principals have guided microbiological research in the identification of the causative agents of infectious diseases often yielding reliable evidence to support the status of a given microbe as a pathogen [1]. These tenets have since been adopted and adapted as canonical best-practice guidelines for proving the role of microorganisms and latterly, genes, in disease [3,4]. More recently, the importance of the commensal gut microbiota for human health has become apparent, with key roles identified for commensals in normal physiological processes from metabolism to immune development and function [5–7].

Studies that apply advanced computational analysis to high-throughput sequencing data from large patient cohorts, or from gnotobiotic mouse models to identify bacteria associated with or enriched in disease, demonstrate the utility and power of the *in silico* approach [8•]. Nevertheless, in response to the ever-mounting correlative evidence suggesting a health-promoting role for various commensal microorganisms, we propose a variant of Koch's postulates (Box 1, Figure 1) that is intended to provide a conceptual framework for experimental validation of the hypotheses inferred from genomic analysis of microbiomes. In addition to the detailed sequence-based microbiota analysis, the proposed postulates advocate for microbial culturing, *in vitro* phenotyping, and *in vivo* models that recapitulate features of human health and disease. The maturation of these methodologies and

Figure 1



The application of Koch's postulates to prove causation in microbiota experiments.
(a) Candidate health-promoting species are detected *in silico* by sequencing and comparing the microbiotas of healthy individuals and those with a specific disease. **(b)** Candidate health-promoting commensal species are isolated and archived as pure cultures in the laboratory. **(c)** The candidate health-promoting commensal ameliorates or mitigates disease when introduced into a diseased individual. **(d)** Following treatment, the health-promoting commensal can be detected in the host to which health was restored.

technologies means that experimentally validated proof of causation now represents an achievable and necessary advancement in gut microbiota research. It is in this context that Koch's postulates, revised for application to microbiota research, are defined and proposed.

First postulate: Identification of the beneficial commensal organism in healthy hosts

The first postulate aligns commensals with health in the same way that pathogens are associated with disease (Box 1). While loss of a commensal species may not cause disease outright, it is possible that the loss of beneficial commensal species could be linked with a predisposition to disease. For example, antibiotic treatment that eliminates commensal organisms would leave the host susceptible to infection with pathogens [8•,9,10]. Shotgun metagenomics datasets representing the microbiomes of healthy humans and of those with particular diseases or predispositions are a logical starting point to assess the candidature of a commensal as a health-promoting microorganism [11].

There are, however, a number of points to consider to enable the realisation of this first postulate. Firstly, a precise and highly resolved taxonomic description of the microorganism of interest is required because fundamentally, health or disease would be caused by species or strains – not by families or genera. For years, researchers of pathogens and probiotics have emphasised the importance of strain-level genomic and phenotypic characterisation [12,13], due to the phenotypic diversity present, even within species [14–16]. Equivalent high-resolution strain characterisation has not been routinely applied to microbiota research, whereas findings attributed to bacterial families and genera are common [17,18]. This is primarily due to the limited taxonomic resolution achievable with the commonly used partial length 16S rRNA gene amplicon sequencing approach. Nevertheless, as a number of studies have successfully demonstrated, it is possible to establish species and strain level conclusions by combining these expert *in silico* analyses with experimental validation [8•,19–21]. These interdisciplinary studies are highly commendable and should come to represent the accepted norm for functional microbiota investigations.

Because the human microbiota is inherently variable between individuals and even within an individual over time [22,23] large datasets are needed to identify robust signals when attempting to address the first postulate. For this reason, in addition to generating new datasets, it will be important to thoroughly interrogate the metagenome datasets that are already in the public domain. Online resources such as EBI-Metagenomics [24], MG-RAST [25], and HPMCD [26] are an obvious starting point for such investigations. It is also essential that the deposited metagenomes are associated with a complete and standardised set of metadata (EBI metagenomics portal; https://www.ebi.ac.uk/training/online/course/ebi-metagenomics-portal-submitting-metagenomics-da/what-are-metadata-and-why-are-they-so-im-0). Such large-scale metagenome investigations are expected to yield evidence supporting the role of various commensal

species in contributing to health. Thus, while deep insights into the potential role of a microorganism in disease can be gleaned from *in silico* analyses, whenever possible published investigations should not stop there, because immense scientific value exists in moving beyond this first postulate.

Second postulate: isolation of the commensal organism as a pure culture

To truly understand a microorganism's biology, we must be able to grow it reliably, in pure culture, under controlled conditions in the laboratory. Although conceptually simple, the routine cultivation of fastidious and anaerobic microbes is, in practice, not trivial. Indeed, the widespread acceptance that many of these microbes were 'unculturable' probably served to discourage many attempts at microbial culturing. Several research teams, including ours, have nevertheless successfully confronted the cultivation issue for the human gut microbiota, albeit with different methodological approaches and outcomes [27–31].

In 2011, Goodman and colleagues used 'high-throughput anaerobic culturing techniques' to generate a 'personal human gut microbiota culture collection' consisting of more than a thousand isolates from one adult human [27]. This was achieved using a most probable number approach, which involved diluting a stool sample to yield empirically clonal cultures, without colony picking. This personal biobank consisted of 1172 taxonomically defined isolates and considerable novelty at the various taxonomic ranks was also captured. By comparing partial length 16S rRNA gene profiling of the original sample and the culture collection, it was inferred that ≈50% of the donor's microbiota, when assessed at the bacterial species level, was arrayed. This was in keeping with the general assessment of the culturability of the gut microbiota of two healthy adults that was estimated at $56 \pm 4\%$ at the species level [27]. Noteworthy aspects of this study include the synergistic integration of culture-independent and culture-dependent techniques, the use of a single set of culture conditions, and the high-throughput approach.

The following year, Lagier *et al.* used 212 different culture conditions and picked 32,500 colonies to isolate 340 bacterial species, 5 fungal species and a giant virus from the stools of three individuals in a process they termed 'culturomics' [28]. In addition to standard cultivation conditions, these researchers used selective, co-culture, and enrichment techniques to increase the recoverable microbial diversity. Taxonomic identification of the picked colonies relied on Matrix-Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) technology. The taxonomy of isolates that were unidentifiable by MALDI-TOF was determined by sequencing the 16S rRNA gene. Whole genome sequences were generated for the 31 new bacterial species isolated.

Various successes and progress in culturing the human gut microbiota were published throughout 2016. We reported that a substantial proportion of the human gut microbiota (234 isolates equalling 134 species, representing 90% of the bacterial abundance at species level) could be cultured on a single bacteriological medium, YCFA [29•]. These isolates and their whole genome sequences were deposited in publically accessible locations, including several international culture collections and online databases, respectively. At around the same time, a number of novel enteric species were recovered by the culturomics approach and genome sequences were generated for many of them [32•]. In addition to demonstrating the general culturability of the gut microbiota, Lau and colleagues [30] developed culture conditions to target species of the Lachnospiraceae family, which comprise a significant unexplored proportion of the healthy human gut microbiota. Cumulatively, these high-throughput approaches for the general and targeted isolation of enteric microorganisms, many of which are novel, is a major boon for microbiology and for microbiota research in particular.

Together, these studies demonstrate that cultivation of the gut microbiota is indeed possible, even with a single set of culture conditions, thereby unlocking the microbiota for phenotypic characterisation and enabling the research community to complement sequence-based predictions with experimental validation.

Third postulate: the commensal strain ameliorates or mitigates disease when introduced into a new host

The availability of pure cultures of the organism of interest and *in vivo* models with readily measurable disease or health-associated indices are essential for fulfilment of the third postulate which states that a microorganism must prevent or mitigate disease when introduced into a new host that would otherwise be susceptible. The biological relevance of the model used must also be carefully considered because it can have a major impact on the types of conclusions that can be drawn and on the translatability of the findings.

For many reasons the mouse has often been the preferred model organism for infection studies with reliable, validated scales and scoring systems for pain and inflammation well established [33–35]. Mouse-specific experimental tools including antibodies, qPCR probes, and ELISA kits, in addition to transgenic and knock-out mice are also widely available. Laboratory mice are typically specific pathogen free (SPF); however, the composition and genetic functionality of the gut microbiota of SPF laboratory mice is considerably different from that of humans

[36•,37,38•]. Moreover, the immune system of SPF mice is less well developed than the immune systems of pet-shop mice [39]. To overcome these limitations, and to use models that are more relevant to the human situation, researchers have been deriving gnotobiotic mice and animals with a human gut microbiota [18,19,40,41] and diet [19,42,43]. Evidently, access to germ-free (GF) mice and to the infrastructure for their husbandry is essential for the use of these specialised models.

Several excellent examples describe the reintroduction of defined mixtures of commensal strains to prevent or reduce infectious diseases by re-establishing the function of colonisation resistance [8•,44•,45,46•,47•,48]. These studies used defined mixtures of human or murine commensal bacteria to prevent *C. difficile* disease prophylactically in standard laboratory mice [47•] or gnotobiotic mice [8•,45] or therapeutically to treat the highly contagious "*C. difficile* supershedding" state in SPF mice [44•]. In a couple of cases, a single bacterium (a Lachnospiraceae isolate [45] or *C. scindens* [8•]) could successfully mitigate disease symptoms in gnotobiotic mice [8•,45]. Moreover, a consortium of six phylogenetically diverse mouse-microbiota isolates effectively resolved disease-associated dysbiosis and the contagiousness of the 'supershedder' state in SPF mice [44•]. These experimental validations are not limited to the study of *C. difficile*; a defined consortium of twelve mouse-bacterial isolates reduced *Salmonella enterica* serovar Typhimurium infection in gnotobiotic mice [46•] while a consortium of four bacterial isolates protected against vancomycin-resistant *Enterococcus faecium* infection in mice [49]. Experimental validations such as these directly support the role of a given strain or defined microbial consortium in re-establishing pathogen colonisation resistance by reducing disease suceptiblity or severity.

Fourth postulate: detection of the commensal strain of interest in a host to which health was restored

A health-associated commensal strain may colonise transiently or establish itself long-term in its intended niche following its re-introduction to a host. The establishment of the commensal in the host would therefore be expected to overcome any prevailing dysbiosis or missing functions by re-establishing colonisation resistance and homeostasis, and contribute to the prevention of disease and restoration of long-term health. This is the principle upon which effective faecal microbiota transplant (FMT) is founded [50•,51].

Rather than performing extensive culturing, it may also be appropriate to use sequencing or molecular methods (for example sequencing or target-specific PCR) or imaging [52] to demonstrate that the exact health-associated commensal colonises the host. This has the advantage of being less labour intensive and more comprehensive than an exclusively culture-based approach. Molecular or sequencing-based detection of specific commensal strains in the post-treatment host would also readily allow for the evaluation of the presence or absence of the target commensal at multiple time-points over a short interval. This could be useful in cases where the beneficial commensal strain is introduced to the host to resolve inflammation or restore gut barrier function, but colonises only transiently. For example, *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* offer a number of desirable therapeutic properties [53,54], though their long-term colonisation of hosts may vary [50•]. Thus, fulfilment of this fourth postulate may encompass culture-based or culture-independent methods.

Outlook

Human microbiome research is poised to stimulate and accelerate biomedical research just as the human genome project did almost two decades ago. Despite the promise of CRISPR-Cas9 systems [55] we currently lack clinically-ready tools to manipulate the human genome. In contrast, the gut microbiota is broadly malleable with dietary interventions, antibiotics, FMT or through emerging, rationally selected live biotherapeutics. The microbiota of other body sites, (*e.g.* skin, mouth) could be similarly manipulated. The identification of such candidate therapeutic microorganisms from large datasets is made possible by high-throughput sequencing combined with sophisticated data analysis, but is also reliant on optimised specimen collection, handling and processing.

By agreeing to adopt a cross-disciplinary research approach, we can realistically look to the future with the intention of translating microbiome science. To do so will inevitably require integration of dry-lab and wet-lab expertise as standard best practise to demonstrate causation in microbiota studies. For it is only by combining sequence-based analyses with comprehensive experimental validation that microbiome research will deliver on its immense potential.

Conflicts of interest

We wish to draw the attention of the Editor to the following facts, which may be considered as potential conflicts of interest to this work. BAN, SCF and TDL are either employees of, or consultants to, Microbiotica.

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