Problems with the concept of gut microbiota dysbiosis

Harald Brüssow*
Laboratory of Gene Technology, Department of Biosystems, KU Leuven, Leuven, Belgium.

Summary
The human microbiome research is with the notable exception of fecal transplantation still mostly in a descriptive phase. Part of the difficulty for translating research into medical interventions is due to the large compositional complexity of the microbiome resulting in datasets that need sophisticated statistical methods for their analysis and do not lend to industrial applications. Another part of the difficulty might be due to logical flaws in terminology particularly concerning ‘dysbiosis’ that avoids circular conclusions and is based on sound ecological and evolutionary reasoning. Many case–control studies are underpowered necessitating more meta-analyses that sort out consistent from spurious dysbiosis–disease associations. We also need for the microbiome a transition from statistical associations to causal relationships with diseases that fulfil a set of modified Koch’s postulates for commensals. Disturbingly, the most sophisticated statistical analyses explain only a small percentage of the variance in the microbiome. Microbe–microbe interactions irrelevant to the host and stochastic processes might play a greater role than anticipated. To satisfy the concept of Karl Popper about conjectures and refutations in the scientific process, we should also conduct more experiments that try to refute the role of the commensal gut microbiota for human health and disease.

Introduction
‘The human microbiota is the focus of one of the most dynamic research fields of our time’ – with this sentence starts a recent Cell review on the human gut microbiome (Schmidt et al., 2018). When looking to the coverage of microbiome research in Nature, Science, Cell and their sister journals, this judgement is by no means an exaggeration. Sometimes entire issues are packed with microbiome papers, like the 30 May 2019 issue of Nature presenting the latest results from the Integrative Human Microbiome Project (HMP) (Proctor and the Integrative HMP (iHMP) Research Network Consortium, 2019).

One of the three subprojects of HMP deals with the gut microbiota in inflammatory bowel disease (IBD), where an impressive array of multomics technologies have been applied to nearly 3000 stool, biopsy and blood samples taken repetitively over one year from 132 patients (Lloyd-Price et al., 2019). Interindividual variation accounted for the majority of the observed variance, while disease status explained only a smaller proportion. During the follow-up, ‘dysbiotic’ gut microbiome excursions occurred in some patients, but were only weakly associated with disease activity. These gut microbiota deviations thus represented potentially stochastic events. Taxonomic shifts to aerotolerant, proinflammatory bacterial clades confirmed observations from previous reports. New are observations of greater gene expression by clostridia and a reduction in an unclassified Subdoligranulum species in IBD. The authors stressed that it is unclear whether multomics microbiome data can predict disease events before their occurrence. Causal analysis needs intervention study designs. The data provide a catalogue of new relationships between multomics features that enable future research, yet microbiome research remains thus largely descriptive.

According to the coordinator of this project, progress in microbiome research has excited industry (Proctor and the Integrative HMP (iHMP) Research Network Consortium, 2019; Proctor, 2019). Financial figures are useful to put the microbiome enterprise into perspective for the microbial biotechnologist. Over the last decade, more than US$ 1.7 billion in research money has been spent on microbiome projects by the public sector. The market
value of microbiome-based products and interventions (diagnostic and therapeutic) is currently estimated to be between US$ 275 and 400 million worldwide and is expected to at least triple over the next years (Proctor and the Integrative HMP (iHMP) Research Network Consortium, 2019; Proctor, 2019). In comparison, the global market for probiotics amounted to US$ 40 billion in 2017 (Reid et al., 2019). Apparently, there are difficulties in translating basic microbiome research into food, nutrition and health or pharmaceutical products. One notable exception is faecal transplantation for the treatment of recurrent *Clostridium difficile* infections (CDI). However, despite clear data on its efficacy (Tariq et al., 2019), its development into a commercial product meets regulatory hurdles (Vyas et al., 2015; Verbeke et al., 2017), and the FDA has recently issued a safety alert pertaining to its use (https://www.fda.gov/vaccines-blood-biologics/safety-availability-biologics/important-safety-alert-regarding-use-fecal-microbiota-transplantation-and-risk-serious-adverse). Furthermore, the basic concept of this approach predates microbiome research (de Vos, 2013), and defined bacterial strains for recurrent CDI (Tvede and Rask-Madsen, 1989; Tvede et al., 2015) still need to be developed into registered products.

**The problem of definitions**

The problems confronting microbiome-derived products for industrial applications are manifold. It might surprise that one hurdle is a question of basic scientific definition, an issue that should not be dismissed as of purely semantic importance. Nearly 2000 years ago, a founding father of medicine stressed the importance of clear scientific terms when writing ‘First, however, we must distinguish and explain clearly the various terms which we are going to use and to what things we apply them; and this will prove not merely an explanation of terms but at the same time a demonstration of the effects of nature’ (Galen, 191 AD). The probiotic field has long discussed what represents a probiotic, and consensus conferences have been organized to find a definition. Having a clear definition does not alone solve scientific questions. In fact, learned societies have only recommended few probiotics for a limited number of health applications (Brüssow, 2019). However, lacking a clear definition of terms risks blurring the discussion.

The microbiome field employs the overarching term ‘dysbiosis’ and its correction to a state of ‘eubiosis’ by targeted interventions. The difference to the probiotic field is that no consensus conference has yet worked out a definition of ‘dysbiosis’ and many microbiome research papers use this term without even an ad hoc definition or specification. This neglect illustrates not only a lack of scientific rigour, but might in fact hamper progress in the microbiome field from a descriptive into a translational science. Over recent years, criticism of the undefined use of the ‘dysbiosis’ term has been repetitively articulated. In a short comment, ‘Dysbiosis is not an answer’, Olesen and Aim (2016) dismissed dysbiosis as a major organizing concept in microbiome science, arguing that it is based on pre-scientific thoughts of microbial imbalances and resembling somewhat the humoral theory of human health. According to these authors, the ambiguity of the definition allows to observe dysbiosis in microbiota composition without actually accomplishing anything scientifically useful. They quote diagnostic microbiome signatures for inflammatory bowel disease (IBD) as an example, which are not superior to the simpler test based on faecal calprotectin measurement. In addition, diagnosing dysbiosis does not tell whether it is a cause or an effect of disease. A detailed historical evaluation of the term dysbiosis and its current use and misuse is provided by Hooks and O’Malley (2017). When they screened microbiota literature, they found ‘imbalance of the microbiota’ as the most common characterization of dysbiosis, with imbalance defined as a loss of homoeostasis, which itself is not, or only rarely, defined, therefore leading to a circular definition in most applications. This vague definition led, in the view of these authors, to a catchall definition lacking scientific value, which has hampered the microbiome field to progress from an association-focused to an explanatory science.

**Approaches to defining dysbiosis**

Levy et al. (2017) distinguished three types of dysbiosis defined as the ‘bloom of pathobionts, loss of commensals, or loss of diversity’, while Vangay et al. (2015) distinguished four dysbiosis types, namely ‘loss of keystone taxa, loss of diversity, shifts in metabolic capacity, or blooms of pathogens’. Pathobionts are defined here as members of the commensal microbiota that have the potential to cause pathology (Chow and Mazmanian, 2010). A keystone species is defined by having a disproportionately large effect on its natural environment relative to its abundance. Many definitions tried to link dysbiosis with disease such as ‘dysbiosis is any change in composition of resident commensals relative to the community found in healthy individuals’ (Petersen and Round, 2014). According to Hooks and O’Malley (2017), this definition pointed to a major methodological problem of the field: by searching for microbiota changes in ill people compared with healthy subjects in case–control studies, the dysbiotic state is tacitly confirmed as conferring illness, which is a classical circular conclusion. Or in a milder form of criticism, Bäckhed et al. (2012) stated that ‘current evidence is insufficient to distinguish
between dysbiosis as a cause or consequence of the disease.

Fundamental criticism on the naïve use of the term dysbiosis was formulated by Levy et al. (2017), who observed that it is not sufficient to compare diseased individuals with a disease-free control cohort; it particularly needs comparisons across different manifestations of the same disease. This request would provide a closer level of association without, however, entirely solving the cause or consequence problem. These authors also called for functional rather than taxonomical interpretations of the dysbiotic microbiota and raised the point of a high contextual dependence of observations, for example with the host immune system acting on the microbiome and the microbiome acting on the host immune system. These authors come with a radical proposal that the definition of a dysbiotic microbiota configuration should fulfill criteria of Koch’s postulates for the definition of a disease-causing microbial agent, here extended for a disease-causing microbiome.

When investigating the link between paediatric dysbiosis and disease, Vangay et al. (2015) mentioned important confounding factors that need to be accounted for in such microbiota–disease analyses, like the temporal maturation of the gut microbiota, and the dependence of the microbiota composition on the type of delivery (Caesarian vs. vaginal) (Chu et al., 2017), on diet (breast- vs. bottle-feeding) and on prior antibiotic use. A detailed description of the gut microbiota from infants taking account of these factors has been provided for healthy infants from Sweden (Bäckhed et al., 2015) and the United States (Baumann-Dudenhoeffer et al., 2018). A single case–control study has documented a microbiota–disease association, namely malnutrition in children from Bangladesh, with the microbiota age development by using machine learning on 16S RNA sequence data that resulted in a ‘relative microbiota maturity index’. The authors found a relative microbiota immaturity for malnourished children (Subramanian et al., 2014). While this observation is of research interest, it is of limited practical importance since this condition can be easily assessed by anthropometry and visual diagnosis.

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mice (Thaiss et al., 2014). If confirmed, such volatility of the gut microbiota constellation would necessitate protocols with standardized stool sampling times (seasons, hours of the day) to support comparisons across studies and allow generalizations to be deduced.

Also, Shade (2017) calls for restraint in using diversity measurement for health assessments: diversity alone has limited value because much context is needed for its interpretation. Discrepancy between different indices of community diversity has long been recognized in the field of ecology, thus complicating comparisons across studies. As such, microbiota diversity is neither good nor bad. The point is easily demonstrated by the human vaginal microbiota, where increased microbial diversity is clearly associated with detrimental effects (bacterial vaginosis, in Afro-American women even associated with compromised birth outcomes; Fettweis et al., 2019). The host genetic background dependency for microbiota–health associations is well demonstrated in vaginal microbiota studies from Afro-American and White US women (Callahan et al., 2017). In the case of the vaginal microbiota, the argument of a co-evolution of a microbiota with the hominid lineage is not applicable since a Lactobacillus-dominated vaginal microbiota is only found in humans and not in apes (Miller et al., 2016).

Underpowered studies and meta-analyses

Many microbiota studies diagnosing dysbiosis in case–control studies are statistically underpowered, counting only small human sample sizes: < 20 subjects per group is not unusual in the field. To account for these limitations, Duvallet (2018) argues for meta-analyses in the dysbiosis field. A meta-analysis can increase the statistical power of many small studies by detecting true signals against a background of false positives. A major asset of meta-analysis is the identification of consistent observations across independent studies or the refutation of spurious associations. A test case is provided for the gut microbiota association with obesity.

Initial studies had reported differences in gut microbial community diversity and differences in the Bacteroidetes/Firmicutes (B/F) ratio among obese and non-obese individuals. A pioneer study was done with small subject numbers (12 obese and 5 lean subjects), showing that the B/F ratio increased with time on a calorie-restricted diet (Ley et al., 2006). A follow-up study in twins enrolling 154 individuals confirmed that obesity is associated with phylum-level changes in the gut microbiota (decreases in Bacteroidetes and increases in Actinobacteria abundance), reduced bacterial diversity and altered bacterial gene expression (Turnbaugh et al., 2009). Together with similar microbiota changes observable in obese and lean mice, the demonstration that the ‘obese’ microbiota had an increased capacity to extract energy from the diet and the demonstration that this trait is transmissible to germ-free mice (Turnbaugh et al., 2006), the obesity–microbiota connection became a showcase for pronounced division-wide changes in microbial gut ecology associated with host pathology. However, a meta-analysis including 10 case–control studies and approximately 2800 subjects (about a third of whom were obese) did not reveal a significant difference in B/F ratio between lean and obese groups and only marginal differences for microbiota diversity (Sze and Schloss, 2016). Finucane et al. (2014) used database mining in the US Human Microbiome Project (HMP) and the European MetaHIT data set and found no associations between B/F ratio and body mass index (BMI) or phylum-level microbiome composition and BMI. The between-study variability in the relative abundance of Bacteroidetes and Firmicutes was greater in their analysis than the within-study differences between lean and obese individuals. In contrast, Falony et al. (2016) found an association, albeit a small one, between microbiome composition and BMI when analysing the gut microbiome in 1106 subjects from the Flemish Gut Flora Project (FGFP). However, based on their data, they estimated that one would need 865 lean and 865 obese volunteers to detect microbiota compositional shifts with a P < 5% significance level and a power of 80%. No single obesity study of this size has yet been conducted.

It would certainly be desirable that future microbiome–disease association studies conduct power calculations with realistically anticipated effect sizes in their published results, as routinely done in the protocols for clinical trials. Such approaches plus meta-analyses will help to distinguish true from spurious associations to allow focusing on promising microbiota–disease associations that lead the field from hype to hope (McKenney & Pamer 2015), with meta-analyses of faecal transfer in C. difficile infection serving as an example (Tariq et al., 2019).

Categorizations of dysbiosis studies

Gilbert et al. (2016) distinguished three categories of microbiota–disease associations: predictable associations (e.g. irritable bowel syndrome, inflammatory bowel syndrome), intriguing associations (e.g. obesity, cardiovascular disease, colon cancer, rheumatoid arthritis) and surprising associations (e.g. major depression, Parkinson disease, autism), which might also represent gradients of likelihood for microbiota being causally involved in diseases or the time needed for translation of research data into clinical practice. Duvallet et al. (2017) used a meta-analysis approach of case–control studies across different disease types that led to interesting conclusions.
They attributed microbiota changes into distinct categories. One category is characterized by an enrichment of a small number of potential pathogens like Fusobacterium, Porphyromonas, Peptostreptococcus, Parvimonas and Enterobacter, as identified in three of four colorectal cancer studies (Wang et al., 2011; Chen et al., 2012; Zeller et al., 2014; Baxter et al., 2016). These studies suggest specific antimicrobials as therapeutic approaches (e.g. bacteriocins or bacteriophages). Another category corresponded to diseases associated with a depletion of health-associated bacteria. For example, five genera from the Ruminococcaceae and Lachnospiraceae families were consistently depleted in IBD patients. These studies suggest probiotic approaches in order to supplement the missing bacteria from the community. A few diseases were characterized by gross changes of microbiota composition, with C. difficile diarrhea being the clearest example, suggesting faecal transplantation as verified by multiple clinical studies. Some microbiota–disease associations were likely due to confounding factors, such as HIV-associated microbiota changes influenced by sex practices or obesity–microbiota associations influenced by diet effects. A few bacteria were apparently non-specifically associated with several diseases: Escherichia/Shigella/Salmonella induced by antibiotic treatments independent of the underlying infection type.

**Eubiosis**

The relatively elusive character of the term ‘dysbiosis’ is the mirror image of the likewise poorly characterized opposite term of ‘eubiosis’. No precise definition has been given for eubiosis beyond a ‘balanced’ microbiota or a microbiota found in healthy subjects. The clear definition of the eubiotic microbiota is particularly important for case–control studies. If the healthy eubiotic microbiota is well defined, both semantically and by its microbiota composition, only microbiota data from a small numbers of control subjects would be needed. This effect is proposed by the ‘Anna Karenina principle’, which states that dysbiotic individuals vary more in microbial community composition than healthy individuals (Zaneveld et al., 2017). The peculiar name of this principle is a pun on a book of Leo Tolstoy, which starts with the sentence: ‘All happy families look alike, each unhappy family is unhappy in its own way’. Support for this principle was found in ocean corals where dysbiotic corals, i.e. stressed corals, have a more variable and unstable microbiome than healthy ones (Ahmed et al., 2019). While the human vaginal microbiota seems to concur with this principle, it is far from clear whether this principle applies to the healthy human gut microbiota (Brüssow, 2016).

If the gut microbiota composition in healthy subjects is highly variable, large numbers of subjects are needed in case–control studies to arrive at significant conclusions. An estimate for the numbers needed can be derived from large microbiome studies of average health individuals from Belgium (Falony et al., 2016) or The Netherlands (Zhemakova et al., 2016). A microbial census yielded about 800 bacterial genera. Yet, the richness of western gut microbiota is still undersampled and the authors estimated that it would need 40 000 subjects to arrive at saturation. The Belgian study yielded 20 core taxa (present in 95% of the samples), and core taxa also belonged to the most abundant taxa. Variation between individuals was in fact substantial, but resulted mainly from changes in the abundance of core taxa like Ruminococcaceae, Bacteroides and Prevotella, all organisms that had previously been proposed as enterotype identifiers (Arumugam et al., 2011). In addition to these three major groups of gut bacteria, a long tail of low abundance bacteria was described that contributed substantially to the functional diversification of the healthy gut microbiota (Arumugam et al., 2011). About 70 factors from the subjects showed significant associations with the microbiota composition, and nearly half of them were also identified in the Dutch cohort as significant covariables (Falony et al., 2016). However, these factors explained a mere 1–15% of the genus abundance variation, therefore suggesting that unknown effects, biotic interactions and even stochastic processes have major influences on the healthy gut microbiota. A strong stochastic element was also manifest in substantial week-to-week gut microbiota changes in healthy children (Sarker et al., 2017a) and day-to-day variation in healthy adults from Bangladesh (Sarker et al., 2012; McCallin et al., 2013). If confirmed by studies from other geographical areas (the high variability of gut microbiota in Bangladesh might reflect a low environmental and food hygiene level), it will be difficult to differentiate dysbiotic microbiome markers from small numbers of controls, particularly if it affects abundance changes in major taxa like the B/F ratio that showed a continuum of variation also in European subjects, as demonstrated by an elderly study from Ireland (Claesson et al., 2011).

**Confounding factors**

Medication for everyday life conditions had the greatest impact on microbiota composition in the Belgian FGGP and the Dutch LifeLines DEEP studies (Falony et al., 2016; Zhemakova et al., 2016). This observation is not surprising in view of the impact of non-antibiotic drugs on commensal bacteria: 24% of 1000 common drugs inhibited bacterial growth *in vitro* (Maier et al., 2018). The strong impact of medication as a confounding factor...
is shown in studies investigating the association of gut microbiota dysbiosis with type 2 diabetes (T2D). A Chinese study described a moderate gut microbiota dysbiosis in a T2D case–control study, where only 4% of the gut microbial genes were associated with T2D. Functional annotation indicated a decrease in butyrate-producing bacteria and an increase in facultative pathogens (Qin et al., 2012). A Danish study associated increases in four Lactobacillus species and decreases in five Clostridium species in women with T2D. Microbial classifiers (Lactobacillus, Akkermansia) differed between the Chinese and Danish T2D cohorts, pointing to population-specific effects (Karlsson et al., 2013). A third study stratified T2D patients according to medication with metformin (Forslund et al., 2015). In metformin-naive patients, a decrease in butyrate-producers was associated with an increase in Lactobacillus with disease. However, in metformin-treated T2D patients, the same authors associated a significant increase of Escherichia with disease, which might explain both the therapeutic and adverse effects (diarrhoea, bloating) of this most widely used anti-diabetic medication. Case–control studies must therefore be stratified for medication in order to provide reliable microbiota–disease associations. Otherwise, microbiota changes could simply be a consequence of disease treatment.

**Multiple omics approaches...**

Due to the substantial interindividual variability and the influence of confounding factors on the gut microbiota, other authors have explored more sophisticated approaches to differentiate healthy from dysbiotic microbiota. One option is to explore the temporal variability with mathematical approaches from theoretical ecology. The two-dimensional parameter space of Taylor's law allowed a definition of a healthy microbiota space from which antibiotic-treated subjects, morbid obese patients and irritable bowel syndrome (IBS) patients could be differentiated (Marti et al., 2017). Gilbert et al. (2016) recommend time-series for microbiome-wide association studies to link microbiota changes to disease. They argue for the establishment of a type of microbial Global Positioning System (GPS), integrating microbiome, host genome and disease subtype differentiation data obtained with multiple omics approaches. In this way, they suggest to follow the microbiome path of at-risk subjects and patients through a principal coordinate analysis (PCoA) plot. Such plots could be of diagnostic use when identifying patients at risk before a disease becomes manifest and would also allow to follow the impact of therapeutic interventions by changes in plot positions. In such a plot analysis, diet interventions considered as mild are supposed to confer small shifts in plot position, while strong (e.g. antibiotics) interventions induce large shifts and game-changing interventions (e.g. faecal transplantation) would lead to ‘teleportation’ of the patient’s microbiota to the healthy area in these plots.

The trend in analysing the healthy gut microbiome and its dysbiotic changes is clearly towards using tools of increasing analytical complexity. One of the latest developments is borrowed from statistical approaches developed to deal with interacting systems of seemingly intractable complexity that was initially applied to the analysis of financial markets (Raman et al., 2019). A large international consortium led by J.I. Gordon, a pioneer of microbiota–obesity and microbiota–malnutrition studies, conducted statistical covariance analysis with stool samples from a Bangladeshi birth cohort, which revealed an ‘ecogroup’ of 15 co-varying bacterial taxa that provide a concise description of the microbiota development in healthy children that was also applicable for children from India and Peru. The primary principal component described 80% of the data variance. The ecogroup analysis allowed a clear differentiation of healthy children from severely malnourished children (SAM) and a weaker differentiation of children with moderate malnutrition (MAM) from healthy controls. Conventional re-feeding therapy caused marked shifts in the principal component analysis (PCA) space of microbiota composition without reaching the PCA position of healthy controls. Aspects of microbe–microbe interaction could be reproduced in a gnotobiotic piglet model.

...and their limitations

Approaches that create a microbial GPS are fascinating, but still far away from translation into clinical practice. The sensitivity and specificity of microbiota tests for disease diagnosis are still relatively unclear. It is also unknown whether microbiota changes are sufficiently fine-grained to allow diagnostically meaningful disease differentiation. More fundamentally, the majority of the gut microbiota studies have been conducted for logistic reasons with stool samples. The microbiota composition of faecal samples differs from that of mucosal samples (Eckburg et al., 2005). Obviously, the mucosal microbiota, due to its closer association with the diseased gut tissues, is more likely to affect disease outcome if the microbiota are drivers of the investigated disease. The faecal microbiota in contrast is a mixture of shed mucosal bacteria and a separate non-adherent luminal microbiota, which is less likely to reflect the disease process. The faecal microbiota is thus potentially only a distant mirror of pathological events, and therefore, we should not expect close correlations with gut disease even if they existed for the mucosal microbiota.
A comprehensive, complex intervention trial

The Gordon-led consortium extended their ecotype analysis (Raman et al., 2019) into a nutritional intervention trial to support the hypothesis that a healthy microbiota development is causally linked to healthy growth (Gehrig et al., 2019). As formulated, this sounds a bit like a circular conclusion. Their starting observation is that malnourished children from Bangladesh showed a delayed maturation of their gut microbiota compared with control children (Subramanian et al., 2014). They designed nutritional components from Bangladesh from microbial inoculation experiments in gnotobiotic mice and piglets that affected a shift from a malnourished to a healthy gut microbiota associated with biomarkers of health and growth in experimental animals. Based on this pre-selection, they conducted a randomized, double-blinded trial with four different feeding regimes in moderately malnourished MAM children (14-17 children per group). The intervention led to a statistically significant, but clinically small, weight increase (weight-per-height Z-scores ameliorated from −2.2 to −1.9) in all four groups. The microbiota composition of the MAM children, which was already close to that of healthy controls, showed a shift towards the healthy gut microbiota composition for the microbiota-directed complementary food 2 (MDCF2) group, who were fed with chickpea, soya and peanut flour plus bananas. In parallel, the authors observed a change to a ‘healthy growth discriminatory’ plasma proteome derived from a comparison of SAM, MAM and healthy children. The data should be interpreted with caution since the intervention was conducted in children with moderate malnutrition who showed already a gut microbiota composition close to healthy children at enrolment. Furthermore, the weight gaining effect was modest and the end-points were biomarkers and not a clinical assessment. One of the significant alterations affected by MDCF2 was a decrease in Bifidobacterium longum. This conclusion is counterintuitive since bifidobacteria are associated with breastfeeding (Simeoni et al., 2016), commonly regarded as desirable infant nutrition, and bifidobacteria are leading probiotic candidate in paediatrics (Brüssow, 2019) and health-associated gut species in elderly (Brüssow, 2013). It remains to be seen whether this microbiota-designed nutritional intervention will have the desired growth effects in future clinical trials. The causal link between this dysbiotic gut microbiota and malnutrition, while perhaps suggestive, is not proven. Proof might need an approach designed according to Koch’s postulates for disease-associated pathogens.

Modified Koch’s postulate for microbiome–health associations

Researchers from the Sanger Institute have suggested postulates for health-associated microbial commensals (Neville et al., 2018). Their first postulate requires that the health-associated commensal is regularly identified in healthy hosts and less frequently in disease hosts. This sounds much like the commonly applied dysbiosis criterion of case–control studies, but differs in important points. Since the human microbiota is highly variable, the authors request large data sets to identify robust signals for fulfilment of this postulate. Furthermore, high-resolution identification at the strain level is needed for this first postulate. Identification at a species level, not to speak of genus or even higher taxonomical levels, is not sufficient. Fulfilment of their second postulate requires pure culture isolation of the identified commensal. Such a request sounded unrealistic. However, the isolation of more than 1000 taxonomically identified bacterial strains from a single human adult stool by the limiting dilution technique (Goodman et al., 2011) demonstrated that such an approach is feasible, albeit labour-intensive. The Sanger scientists demonstrated that 234 isolates representing 134 species and corresponding to 90% of the abundance of stool bacteria at species level could be cultivated even when using a single medium (Browne et al., 2016). Their third postulate stipulates that the commensal strain(s) ameliorates disease when introduced into a new host. The in vivo model should be a biologically relevant vertebrate model. Mice are commonly used for this demonstration, where mixtures of commensals (Lawley et al., 2012) and even single commensal strains (Buffie et al., 2015) showed prophylactic and therapeutic activity against C. difficile or Salmonella enterica infections (Brugiroux et al., 2016). As for the original Koch’s third postulate, this criterion is a work-intensive, but necessary approach that is complicated by biological intricacies of the mouse model that might limit extension to the human condition and thus translational research (Arrieta et al., 2016). The fourth postulate requires that the commensal strain can be detected following its introduction into a host who experienced health amelioration. Since this detection can be done by PCR, this criterion seems highly feasible, but caveats exist because commensals might not need to persist in order to mediate a therapeutic effect if they downregulate inflammatory processes or increase gut barrier functions and become thereafter dispensable (Li et al., 2016), as postulated for some probiotics.

When applying Koch-type postulates for the commensal–health, and by extension to the dysbiosis–disease, connection, one realizes how far we are still from a clear definition of the field, beyond the possible exception of commensals and C. difficile infection. However, in that area of clear clinical benefit by faecal transplantation, we are, except for an approach predating the microbiome era (Tvæde and Rask-Madsen, 1989; Tvæde et al., 2015), not yet at an intervention level with microbial elements defined at the strain level.
On conjectures and refutations

The concept of eubiosis is linked to another concept, that of a holobiont or a hologenome (the collective genome of host and microbiome). Proponents of this concept state that all animals and plants establish symbiotic relationships with microorganisms, which are transmitted between generations and affect the fitness of the holobiont within its environment, thus leading to a type of superorganism and a new unit of selection in evolution (Zilber-Rosenberg and Rosenberg, 2008). This concept seems to influence many microbiologists with the tacit assumption that a disturbance on the microbiome side (dysbiosis) should lead to fitness loss of the holobiont and to health disturbances in pronounced cases in the animal host. Evolutionary biologists are rather sceptical about this concept, not least because it introduces Lamarckian elements into Darwinian evolution. Moran and Sloan (2015) argued that the hypothesis stating that host-specific microbial community compositions have evolved for the benefit of the host should not be accepted as the null hypothesis for explaining features of host–symbiont associations. While this can be the case, for example, in corals (which show, however, a much more intimate animal–microbe relationship), this case cannot be generalized. Conflicts between hosts and their associated microbes are common even with the closest imaginable host–microbe (mitochondria) interaction resulting in cytonuclear conflicts. Many more situations in the gut will, in addition, reflect microbe–microbe conflicts without effects on the host. These unseen intermicrobial conflicts might suggest a high influence of stochastic processes in human gut microbiome compositions (Falony et al., 2016, Zhernakova et al., 2016). Instead of anticipating without further proof the detrimental effects of microbial dysbiosis on human health while they reflect only inner-microbial competition, we should look for cases that fulfill the modified Koch’s postulates for microbial commensals that mediate human health. Otherwise, the dysbiosis – like the holobiont – concept risks to cause more confusion than clarity. The philosopher Popper (2002) has stated that knowledge acquisition, and thus also scientific research, consists of the dialectic process of conjectures on the one side and refutations on the other side. Conjectures are manifold in the microbiome field, giving the impression that we are at the threshold of a ‘New Biology’. Instead of creating more and more thrilling conjectures in that field, not only should we look for putting them on a firm conceptual ground with modified Koch-like postulates, we should also actively seek refutations of the microbiome working hypotheses. The refutation arm of knowledge building is currently underrated in the scientific community. High-impact journals and grant agencies look more for stimulating new conjectures than for down to the earth refutations of them. However, this unequal rating of the two arms of knowledge building leads to a serious underuse of the refutation arm for the advancement of knowledge.

Experiences with diarrhoea

Instead of ending with this rather theoretical outlook, I will shortly mention our own experiences in the dysbiosis–health and disease field. When we tried to treat children suffering from *E. coli* diarrhoea with coliphages (phage therapy) in Bangladesh, we realized that the faecal microbiome of the patients displayed a marked dysbiosis with increased streptococcal abundance compared with local healthy control children (Sarker et al., 2016). *E. coli* titres were not prominent and did not correlate with quantitative diarrhoea parameters, while stool streptococci did. With recovery from diarrhoea, the streptococcal abundance decreased and the faecal microbiota approached that of control children. It was thus tempting to associate the faecal streptococcal abundance increase with diarrhoea. However, the streptococci belonged to two commensal groups (*S. salivarius* and *S. bovis* species complex) and genome sequences from stool isolates showed no virulence factors (Sarker et al., 2016). In subsequent studies, we found the same faecal streptococcal abundance increase in diarrhoea patients irrespective of diarrhoea aetiology, even including patients with rotavirus, a clearly defined paediatric diarrhoea pathogen (Kieser et al., 2018). When correcting for stool bacterial counts and stool volumes, quantitatively determined streptococcal stool output was only weakly increased compared with control children. An increase in relative abundance should always be corrected for total bacterial counts: a trivial, but essential control (Vandeputte et al., 2017) neglected in many microbiome studies. The streptococcal abundance increase and its apparent association with diarrhoea might simply be a consequence of the elimination of the typical colon microbiota by the watery diarrhoea pathology (purging), leading to a relative prominence of faecal streptococci, which are indeed commensals of the small intestine and perhaps less affected by gut emptying since the peristaltic flow in the small intestine is anyways high (Brüssow, 2016). In two other special groups of diarrhoea patients (maltreated children with acute diarrhoea, children with persistent diarrhoea), we observed another faecal microbiota dysbiosis (Kieser et al., 2017, Sultana, submitted). This time, we observed an *E. coli* increase documented with both abundance increase and absolute titre increase compared with control children. While *E. coli* could be a pathogen for these forms of diarrhoea (Sarker et al., 2017b), metagenome
sequencing did not suggest diarrhoea-specific virulence gene increases in the stools of patients. Based on clinical data and evidence from mouse models (Faber et al., 2016), it seems more likely that their increase is a consequence of treatment with antibiotics. In all of these cases, the diarrhoea–dysbiosis association is more likely to represent a consequence rather than a cause for diarrhoea. Also, the observation that the dysbiosis ameliorates with recovery from diarrhoea is not a strong argument for a causal association.

Acute diarrhoea is an interesting test case for microbiome research since the disease is of short duration and self-limiting. It represents a natural perturbation of the physiological gut microbiota equilibrium and its disturbance by the pathological process and the re-establishment of a new equilibrium holds promises to understand the mechanisms of microbe–microbe interaction in the gut.

**Outlook**

Medical interventions, like antibiotic treatment or gut cleansing in preparation for colonoscopy represent interesting research opportunities for microbiome analyses since all enrolled subjects are informative and biological samples can be easily obtained before, during and after interventions (Fukuyama et al., 2017). These data can complement the insights from acute diarrhoea studies.

A critical test for a causative effect of dysbiosis on disease can only be provided by prospective studies where microbiota composition is regularly established for all participants before diseases are occurring. Such approaches are labour-intensive and costly, even for diseases occurring with high frequency, like diarrhoea in children from developing countries. If the suspected disease-causing dysbiosis is then observed in subjects before they develop the manifest disease, but not in age- and milieu-matched children, who do not develop the specified disease, a causative role can be reasonably anticipated. A definitive proof will still need evidence for a mechanism of action by the dysbiosis. We are currently conducting a birth cohort study in Bangladesh with nearly 300 children who are clinically followed over a 2-year observation period combined with regular microbiota sampling. Longitudinal studies and studies that fulfil the ‘commensal Koch’s postulates’ are needed to put the gut microbiota dysbiosis connections with human health and disease on a firm scientific basis. Such claims might sound like scientific rigourism since it implicates many years of further research. However, we should be aware of what is needed for a proof in the dysbiosis/microbiome field, so as not to be tempted into premature conclusions and thus creating unrealistic hopes for translation of microbiome research into amelioration of human health.

Clinical intervention trials are finally the critical test for the practical value of microbiome research. The success of faecal transplantation in *C. difficile* infection is a sign of hope (Tariq et al., 2019), but we need to decipher how it works mechanistically, both to achieve an industrial product and to understand why faecal transplantation works less well in other gastroenterology diseases (Imdad et al., 2018).

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**Conflict of interest**

None declared.

**References**

Ahmed, H.I., Herrera, M., Liew, Y.J., and Aranda, M. (2019) Long-term temperature stress in the coral model *Aiptasia* supports the “anna karenina principle” for bacterial microbiomes. *Front Microbiol* 10: 975. https://doi.org/10.3389/fmicb.2019.00975

Arrieta, M.C., Walter, J., and Finlay, B.B. (2016) Human microbiota-associated mice: a model with challenges. *Cell Host Microbe* 19: 575–578. https://doi.org/10.1016/j.chom.2016.04.014

Arunugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R., et al. (2011) Enterotypes of the human gut microbiome. *Nature* 473: 174–180. https://doi.org/10.1038/nature09944.

Backhed, F., Fraser, C.M., Ringel, Y., Sanders, M.E., Sartor, R.B., Sherman, P.M., et al. (2012) Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host Microbe* 12: 611–622. https://doi.org/10.1016/j.chom.2012.10.012

Backhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015) Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* 17: 690–703. https://doi.org/10.1016/j.chom.2015.05.012

Baumann-Dudenhoeffer, A.M., D’Souza, A.W., Tarr, P.I., Warner, B.B., and Dantas, G. (2018) Infant diet and maternal gestational weight gain predict early metabolic maturation of gut microbiomes. *Nat Med* 24: 1822–1829. https://doi.org/10.1038/s41591-018-0216-2

Baxter, N.T., Ruffin, M.T., Rogers, M.A., and Schloss, P.D. (2016) Microbiota-based model improves the sensitivity of fecal immunochemical test for detecting colonic lesions. *Genome Med* 8: e37.

Browne, H.P., Forster, S.C., Anonye, B.O., Kumar, N., Neville, B.A., Stares, M.D., et al. (2016) Culturing of ‘unculturable’ human microbiota reveals novel taxa and extensive sporulation. *Nature* 533: 543–546. https://doi.org/10.1038/nature17645

Brugiroux, S., Beutler, M., Pfann, C., Garzetti, D., Ruscheweyh, H.J., Ring, D., et al. (2016) Genome-guided design
of a defined mouse microbiota that confers colonization resistance against *Salmonella enterica* serovar Typhimurium. *Nat Microbiol* 2: 16215. https://doi.org/10.1038/s41564-019-0456-2

Brüssow, H. (2013) Microbiota and healthy ageing: observational and nutritional intervention studies. *Microb Biotechnol* 6: 326–334. https://doi.org/10.1111/1751-7915.12048

Brüssow, H. (2016) How stable is the human gut microbiota? And why this question matters. *Environ Microbiol* 18: 2779–2783. https://doi.org/10.1111/1462-2920.13473

Brüssow, H. (2019) Probiotics and prebiotics in clinical tests: an update. *F1000Research* 8(F1000 Faculty Rev): 1157.

Buffie, C.G., Bucci, V., Stein, R.R., McKenney, P.T., Ling, L., Gobourne, A., et al. (2015) Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. *Nature* 517: 205–208. https://doi.org/10.1038/nature13828

Callahan, B.J., DiGiulio, D.B., Goldtsman, D.S.A., Sun, C.L., Costello, E.K., Jeganathan, P., et al. (2017) Replication and refinement of a vaginal microbial signature of preterm birth in two racially distinct cohorts of US women. *Proc Natl Acad Sci USA* 114: 9966–9971. https://doi.org/10.1073/pnas.1705891114

Chen, W., Liu, F., Ling, Z., Tong, X., and Xiang, C. (2012) Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PLoS ONE* 7: e39743.

Chow, J., and Mazmanian, S.K. (2010) A pathobiont of the microbiota balances host colonization and intestinal inflammation. *Cell Host Microbe* 7: 265–276. https://doi.org/10.1016/j.chom.2010.03.004

Chu, D.M., Ma, J., Prince, A.L., Antony, K.M., Seferovic, M.D., and Aagaard, K.M. (2017) Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. *Nat Med* 23: 314–326. https://doi.org/10.1038/nm.4272

Claesson, M.J., Cusack, S., O’Sullivan, O., Greene-Diniz, R., de Weerd, H., Flannery, E., et al. (2011) Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc Natl Acad Sci USA* 108(Suppl 1): 4586–4591. https://doi.org/10.1073/pnas.100097107

Coyte, K.Z., Schluter, J., and Foster, K.R. (2015) The ecology of the microbiome: networks, competition, and stability. *Science* 350: 663–666. https://doi.org/10.1126/science.aad2602

Duvall et al. (2018) Meta-analysis generates and prioritizes hypotheses for translational microbiome research. *Microb Biotechnol* 11: 273–276. https://doi.org/10.1111/1751-7915.13047

Duvall, C., Gibbons, S.M., Gurry, T., Irizarry, R.A., and Alm, E.J. (2017) Meta-analysis of gut microbiome studies identifies disease-specific and shared responses. *Nat Commun* 8: 1784. https://doi.org/10.1038/s41467-017-01973-8

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005) Diversity of the human intestinal microbial flora. *Science* 308: 1635–1638.

Faber, F., Tran, L., Byndloss, M.X., Lopez, C.A., Velazquez, E.M., Kerrinnes, T., et al. (2016) Host-mediated sugar oxidation promotes post-antibiotic pathogen expansion. *Nature* 534: 697–699.

Falony, G., Joossens, M., Vieira-Silva, S., Wang, J., Darzi, Y., Faust, K., et al. (2016) Population-level analysis of gut microbiome variation. *Science* 352: 560–564. https://doi.org/10.1126/science.aad3503.

Fettweis, J.M., Serrano, M.G., Brooks, J.P., Edwards, D.J., Girerd, P.H., Parikh, H.I., et al. (2019) The vaginal microbiome and preterm birth. *Nat Med* 25: 1012–1021. https://doi.org/10.1038/s41591-019-0450-2

Finucane, M.M., Sharpton, T.J., Laurent, T.J., and Pollard, K.S. (2014) A taxonomic signature of obesity in the microbiome? Getting to the guts of the matter. *PLoS ONE* 9: e84689. https://doi.org/10.1371/journal.pone.0084689.

Forslund, K., Hildebrand, F., Nielsen, T., Falony, G., Le Chatelier, E., Sunagawa, S., et al. (2015) Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature* 528: 262–266. https://doi.org/10.1038/nature15766.

Galen (about AD 190). On the natural faculties. In *Encyclopaedia Britannica. The Great Books Volume 9*. Chicago, IL: University of Chicago, 1992, p. 347.

Gehrig, J.L., Venkatesh, S., Chang, H.W., Hibberd, M.C., Kung, V.L., Cheng, J., et al. (2019) Effects of microbiota-directed foods in gnotobiotic animals and undernourished children. *Science* 365:pii: eaau4732. https://doi.org/10.1126/science.aau4732.

Gilbert, J.A., Quinn, R.A., Debelius, J., Xu, Z.Z., Morton, J., Garg, N., et al. (2016) Microbiome-wide association studies link dynamic microbial consortia to disease. *Nature* 535: 94–103. https://doi.org/10.1038/nature18850.

Goodman, A.L., Kallstrom, G., Faith, J.J., Reyes, A., Moore, A., Dantas, G., and Gordon, J.I. (2011) Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. *Proc Natl Acad Sci USA* 108: 6252–6257. https://doi.org/10.1073/pnas.1102938108

Hooks, K.B. and O’Malley, M.A. (2017) Dysbiosis and its discontents. *MBio* 8: pii: e01492-17. https://doi.org/10.1128/mbio.e01492-17

Imdad, A., Nicholson, M.R., Tanner-Smith, E.E., Zackular, J.P., Gomez-Duarte, O.G., Beaulieu, D.B., and Acra, S. (2018) Fecal transplantation for treatment of inflammatory bowel disease. *Cochrane Database Syst Rev* 11: CD012774. https://doi.org/10.1002/14651858.CD012774.pub2

Jha, A.R., Davenport, E.R., Gautam, Y., Bhandari, D., Dantkar, S., Ng, K.M., et al. (2018) Gut microbiome transition across a lifestyle gradient in Himalaya. *PLoS Biol* 16: e2005396. https://doi.org/10.1371/journal.pbio.2005396

Johnson, K.V., and Burnet, P.W. (2016) Microbiome: should we diversify from diversity? *Gut Microbes* 7: 455–458.

Karlsson, F.H., Tremaroli, V., Nookaew, I., Bergström, G., Behre, C.J., Fagerberg, B., et al. (2013) Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature* 498: 99–103. https://doi.org/10.1038/nature12198.

© 2019 The Authors. *Microbial Biotechnology* published by John Wiley & Sons Ltd and Society for Applied Microbiology. *Microbial Biotechnology*, 13, 423–434
Kieser, S., Sarker, S.A., Berger, B., Sultana, S., Chisti, M.J., Islah, S.B., et al. (2017) Antibiotic treatment leads to fecal *Escherichia coli* and coliphage expansion in severely malnourished diarrhea patients. *Cell Mol Gastroenterol Hepatol* 5: 458–460.e6. https://doi.org/10.1016/j.cemgh.2017.11.014

Kieser, S., Sarker, S.A., Sakwinska, O., Foata, F., Sultana, S., Khan, Z., et al. (2018) Bangladeshi children with acute diarrhoea show faecal microbiomes with increased *Streptococcus* abundance, irrespective of diarrhoea aetiology. *Environ Microbiol* 20: 2256–2269. https://doi.org/10.1111/1462-2920.12474

Lawley, T.D., Clare, S., Walker, A.W., Stares, M.D., Connor, T.R., Raisen, C., et al. (2012) Targeted restoration of the intestinal microbiota with a simple, defined bacteriother-apy resolves relapsing *Clostridium difficile* disease in mice. *PLoS Pathog* 8: e1002995. https://doi.org/10.1371/journal.ppat.1002995

Levy, M., Kołodziejczyk, A.Y., Thais, C.A., and Elinav, E. (2017) Dysbiosis and the immune system. *Nat Rev Immunol* 17: 219–232. https://doi.org/10.1038/nri.2017.7

Ley, R.E., Turnbaugh, P.J., Klein, S., and Gordon, J.I. (2006) Microbial ecology: human gut microbes associated with obesity. *Nature* 444: 1022–1023.

Li S.S., Zhu, A., Benes, V., Costea, P.I., Hercog, R., Hildebrand, F., et al. (2016) Durable coexistence of donor and recipient strains after fecal microbiota transplantation. *Science* 352: 586–589. https://doi.org/10.1126/science.aaad8852

Lloyd-Price, J., Arze, C., Ananthakrishnan, A.N., Schirmer, M., Avila-Pacheco, J., Poon, T.W., et al. (2019) Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* 569: 655–662. https://doi.org/10.1038/s41586-019-1237-9

Maier, L., Pruteanu, M., Kuhn, M., Zeller, G., Telzerow, A., Anderson, E.E., et al. (2018) Extensive impact of non-antibiotic drugs on human gut microbiota. *Nature* 555: 623–628. https://doi.org/10.1038/nature25979

Martí, J.M., Martínez-Martínez, D., Rubio, T., Gracia, C., Peña, M. and Latorre, A., et al. (2017) Health and disease imprinted in the time variability of the human microbiome. *mSystems* 2: pii: e01144-16. https://doi.org/10.1128/mSystems.01144-16.

McCallin, S., Sarker, A.S., Barretto, C., Sultana, S., Berger, B., Huq, S., et al. (2013) Safety analysis of a Russian phage cocktail: from metagenomic analysis to oral application in healthy human subjects. *Virology* 443: 187–196. https://doi.org/10.1016/j.virol.2013.05.022

McKenney, P.T., and Pamer, E.G. (2015) From hype to hope: the gut microbiota in enteric infectious disease. *Cell* 163: 1326–1332. https://doi.org/10.1016/j.cell.2015.

Miller, E.A., Beasley, D.E., Dunn, R.R., and Archie, E.A. (2016) Lactobacilli dominance and vaginal pH: why is the human vaginal microbiome unique? *Front Microbiol* 7: 1936. https://doi.org/10.3389/fmicb.2016.01936

Moeller, A.H., Caro-Quintero, A., Mjungu, D., Georgiev, A.V., Lonsdorf, E.V., Muller, M.N., et al. (2016) Cospeciation of gut microbiota with hominids. *Science* 353: 380–382. https://doi.org/10.1126/science.aaf9351

Moran, N.A., and Sloan, D.B. (2015) The Hologenome Concept: Helpful or Hollow? *PLoS Biol* 13: e1002311. https://doi.org/10.1371/journal.pbio.1002311.

Neville, B.A., Forster, S.C., and Lawley, T.D. (2018) Commensal Koch’s postulates: establishing causation in human microbiota research. *Curr Opin Microbiol* 42: 47–52. https://doi.org/10.1016/j.mib.2017.10.001

Olesen, S.W., and Alm, E.J. (2016) Dysbiosis is not an answer. *Nat Microbiol* 1: 16228. https://doi.org/10.1038/nmicrobiol.2016.228

Petersen, C., and Round, J.L. (2014) Defining dysbiosis and its influence on host immunity and disease. *Cell Microbiol* 16: 1024–1033. https://doi.org/10.1111/cmi.12308

Popper, K. (2002) Conjectures and Refutations. *The Growth of Scientific Knowledge*. New York, NY: Routledge Classics. Taylor and Francis Group, London and New York.

Proctor, L.M. and the Integrative HMP (iHMP) Research Network Consortium (2019) The integrative human microbiome project. *Nature* 569: 641–648. https://doi.org/10.1038/s41586-019-1238-8

Proctor, L. (2019) What’s next for the human microbiome? *Nature* 569: 623–625.

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., et al. (2012) A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490: 55–60. https://doi.org/10.1038/nature11450.

Raman, A.S., Gehrig, J.L., Venkatesh, S., Chang, H.W., Hibberd, M.C., Subramanian, S., et al. (2019) A sparse covarying unit that describes healthy and impaired human gut microbiota development. *Science* 365: pii: eaau4735. https://doi.org/10.1126/science.aau4735.

Reid, G., Gadir, A.A., and Dhir, R. (2019) Probiotics: reiterating what they are and what they are not. *Front Microbiol* 10: 424.

Sarker, S.A., McCallin, S., Barretto, C., Berger, B., Pittet, A.C., Sultana, S., et al. (2012) Oral T4-like phage cocktail application to healthy adult volunteers from Bangladesh. *Virology* 434: 222–232. https://doi.org/10.1016/j.virol.2012.09.002

Sarker, S.A., Sultana, S., Reuteler, G., Moine, D., Combres, P., Charton, F., et al. (2016) Oral phage therapy of acute bacterial diarrhea with two coliphage Preparations: a randomized trial in children from Bangladesh. *EBioMedicine* 4: 124–137. https://doi.org/10.1016/j.ebiom.2015.12.023

Sarker, S.A., Berger, B., Deng, Y., Kieser, S., Foata, F., Moine, D., et al. (2017a) Oral application of *Escherichia coli* bacteriophage: safety tests in healthy and diarrheal children from Bangladesh. *Environ Microbiol* 19: 237–250. https://doi.org/10.1111/1462-2920.13574

Sarker, S.A., Ahmed, T., and Brüssow, H. (2017b) Persistent diarrhea: a persistent infection with enteropathogens or a gut commensal dysbiosis? *Environ Microbiol* 19: 3789–3801. https://doi.org/10.1111/1462-2920.13873

Schmidt, T.S.B., Raes, J., and Bork, P. (2018) The human gut microbiome: from association to modulation. *Cell* 172: 1199–1215. https://doi.org/10.1016/j.cell.2018.02.044.

Schnorr, S.L., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia, G., et al. (2014) Gut microbiome of the Hadza hunter-gatherers. *Nat Commun* 5: 3654. https://doi.org/10.1038/ncomms4654

Shade, A. (2017) Diversity is the question, not the answer. *ISME J* 11: 1–6. https://doi.org/10.1038/ismej.2016.118

Simeoni, U., Berger, B., Junick, J., Blaut, M., Pecquet, S., Rezzonico, E., et al. (2016) Gut microbiota analysis
reveals a marked shift to bifidobacteria by a starter infant formula containing a symbiotic of bovine milk-derived oligosaccharides and *Bifidobacterium animalis* subsp. lactis CNCM I-3446. *Environ Microbiol* **18**: 2185–2195. https://doi.org/10.1111/1462-2920.13144

Smits, S.A., Leach, J., Sonnenburg, E.D., Gonzalez, C.G., Lichtman, J.S., Reid, G., *et al.* (2017) Seasonal cycling in the gut microbiome of the Hadza hunter-gatherers of Tanzania. *Science* **357**: 802–806. https://doi.org/10.1126/science.aan4834

Subramanian, S., Huq, S., Yatsunenko, T., Haque, R., Mahfuz, M., Alam, M.A., *et al.* (2014) Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature* **510**: 417–421. https://doi.org/10.1038/nature13421

Sze, M.A. and Schloss, P.D. (2016) Looking for a signal in the noise: revisiting obesity and the microbiome. *MBio* **7**: pii: e01018-16. https://doi.org/10.1128/mbio.01018-16

Tariq, R., Pardi, D.S., Bartlett, M.G., and Khanna, S. (2019) Low cure rates in controlled trials of fecal microbiota transplantation for recurrent *Clostridium difficile* infection: a systematic review and meta-analysis. *Clin Infect Dis* **68**: 1351–1358. https://doi.org/10.1093/cid/ciy721

Thaiss, C.A., Zeevi, D., Levy, M., Zilberman-Schapira, G., Suez, J., Tengelder, A.C., *et al.* (2014) Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. *Cell* **159**: 514–529. https://doi.org/10.1016/j.cell.2014.09.048

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., and Gordon, J.I. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**: 1027–1031.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., *et al.* (2009) A core gut microbiome in obese and lean twins. *Nature* **457**: 480–484. https://doi.org/10.1038/nature07540

Tvede, M., and Rask-Madsen, J. (1989) Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhoea in six patients. *Lancet* **1**: 1156–1160.

Tvede, M., Tinggaard, M., and Helms, M. (2015) *Rectal bacteriotherapy for recurrent Clostridium difficile*-associated diarrhoea: results from a case series of 55 patients in Denmark 2000-2012. *Clin Microbiol Infect* **21**: 48–53. https://doi.org/10.1016/j.cmi.2014.07.003

Vandeputte, D., Kathagen, G., D'hoe, K., Vieira-Silva, S., Valles-Colomer, M., Sabino, J., *et al.* (2017) Quantitative microbiome profiling links gut community variation to microbial load. *Nature* **551**:507–511. https://doi.org/10.1038/nature24460

Vangay, P., Ward, T., Gerber, J.S., and Knights, D. (2015) Antibiotics, pediatric dysbiosis, and disease. *Cell Host Microbe* **17**: 553–564. https://doi.org/10.1016/j.chom.2015.04.006

Verbeke, F., Janssens, Y., Wynendaele, E., and De Spiegeleer, B. (2017) Faecal microbiota transplantation: a regulatory hurdle? *BMC Gastroenterol* **17**: 128. https://doi.org/10.1186/s12876-017-0687-5

de Vos, W.M. (2013) Fame and future of faecal transplantations—developing next-generation therapies with synthetic microbiomes. *Microb Biotechnol* **6**: 316–325. https://doi.org/10.1111/1751-7915.12047

Vyas, D., Aekka, A., and Vyas, A. (2015) Fecal transplant policy and legislation. *World J Gastroenterol* **21**: 6–11. https://doi.org/10.3748/wjg.v21.i1.6

Wang, T., Cai, G., Qiu, Y., Fei, N., Zhang, M., Pang, X., *et al.* (2011) Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *ISME J* **6**: 320–329.

Zaneveld, J.R., McMinds, R., and Vega Thurber, R., (2017) Stress and stability: applying the Anna Karenina principle to animal microbiomes. *Nat Microbiol* **2**: 17121. https://doi.org/10.1038/s41564-017-0032-z

Zeller, G., Tap, J., Voigt, A.Y., Sunagawa, S., Kultima, J.R., Costea, P.I., *et al.* (2014) Potential of fecal microbiota for early-stage detection of colorectal cancer. *Mol Syst Biol* **10**: 766.

Zhernakova, A., Kurilshikov, A., Bonder, M.J., Tigchelaar, E.F., Schirmer, M., Vatanen, T., *et al.* (2016) Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. *Science* **352**: 565–569. https://doi.org/10.1126/science.aad3369.

Zilber-Rosenberg, I., and Rosenberg, E. (2008) Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol Rev* **32**: 723–735. https://doi.org/10.1111/j.1574-6976.2008.00123.x