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Microbiota-gut-brain research: A critical analysis

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Abstract

Microbiota-gut-brain (MGB) research is a fast-growing field of inquiry with important implications for how human brain function and behaviour are understood. Researchers manipulate gut microbes ("microbiota") to reveal connections between intestinal microbiota and normal brain functions (e.g., cognition, emotion, and memory) or pathological states (e.g., anxiety, mood disorders, and neural developmental disorders such as autism). Many claims are made about causal relationships between gut microbiota and human behaviour. By uncovering these relationships, MGB research aims to offer new explanations of mental health and potential avenues of treatment.

So far, limited evaluation has been made of MGB's methods and its core experimental findings, many of which are extensively reiterated in copious reviews of the field. These factors, plus the self-help potential of MGB, have combined to encourage uncritical public uptake of MGB discoveries. Both social and professional media focus on the potential for dietary intervention in mental health, and causal relationships are assumed to be established.

Our target article has two main aims. One is to examine critically the core practices and findings of experimental MGB research and to raise questions about them for brain and behavioural scientists who may not be familiar with the field. The other is to challenge the way in which MGB findings are presented. Our positive goal is to suggest how current problems and weaknesses may be addressed, in order for both scientific and public audiences to gain a clearer picture of MGB research and its strengths and limitations.

1. Introduction

A growing body of "microbiome" research is investigating microbially mediated connections between the gut and brain. Microbes in the gut ("microbiota") apparently have effects on how humans think, perceive, and experience the world. Numerous scientific articles stress how this research is "revolutionary" and "paradigm-shifting" (e.g., Liu 2017; Mayer et al. 2014). Although such hyperbole is characteristic of microbiome research more generally, many basic views about human capacities are challenged by suggestions that gut microbiota are causally influencing brains and behaviour.

Microbiota-gut-brain (MGB) researchers seek to explain and treat behavioural, cognitive, and mood disorders in host organisms, including humans. The basic methodology is to alter the gut microbiota in rodents, or compare the behaviour of animals with and without microbiota. Some interpretations of the findings from such studies make quite radical claims about the nature of our relationship with our microorganisms and the extent of their control over us. These interpretations propose new ways in which common psychiatric and psychological disorders can be treated, and even normal cognition enhanced. Not surprisingly, these sorts of claims about microbiota and gut-brain connections are of broad interest and have received a great deal of attention in the wider public sphere. Although a critical literature is beginning to develop on both microbiome research generally (Bik 2016; Hanage 2014; Quigley 2017) and MGB research in particular (e.g., Bruce-Keller et al. 2018; Forsythe et al. 2016), a systematic scrutiny from outside of the field has yet to be conducted.

Our aim is to investigate MGB claims and the research that lies behind them. To do this, we focus on the field's 25 most cited experimental papers of the last decade. We analyse first the methodologies underpinning these core studies, and then their findings, before contextualizing these papers within the wider MGB literature. Our conclusions are cautionary and have a constructive aim. Despite the rapidly increasing body of work in the MGB area, and the wide audiences it reaches, even the most cited papers are at best suggestive. Both methodological and

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interpretative aspects of this research require consolidation and greater depth. We discuss this message and its broader implications for brain and behavioural research, as well as its communication to a wider audience.

2. Context and historical background

MGB research weaves together several strands of earlier investigation from neuroscience, gastroenterology, and microbiology. The exploration of connections between the gut and brain has a particularly long and venerable research history. Early psychologists William James and Carl Lange are seen as forerunners of brain-gut-axis research (e.g., Eisenstein 2016), although they made limited claims about these connections. James merely insisted that "visceral stirrings" should be conceptualized as part of the emotion of fear (1884). Subsequent research continued to connect emotional responses to visceral signals. In the early twentieth century, for example, Walter Bradford Cannon observed that "the movements of the stomach immediately stopped" when "a female [cat] with kittens turned from her state of quiet contentment to one of apparent restlessness" (Cannon 1909, p. 484). He postulated that these changes depended on the sympathetic nerve supply (Cannon 1911).

More fine-grained studies followed. The administration of adrenaline, which is released by the host after activation of the sympathetic nervous system, was discovered to lower the number of pathogenic bacteria needed to establish a generalized infection or to kill the animal (Evans et al. 1948; Renaud & Miget 1930). Although these effects were attributed to decreased recruitment of white blood cells (Evans et al. 1948), it was realized much later that adrenaline also diminishes the bactericidal activity of these cells (Qualliotine et al. 1972). Other experiments revealed that adrenaline actually reduces host mortality after the injection of bacterial toxins (Chedid & Boyer 1953; Hodoval et al. 1968), which suggested that this hormone has different effects on living bacteria and bacterial fragments. Another molecule, acetylcholine, which is released by the parasympathetic nervous system of animals, was also shown by other research to be produced by a strain of the bacterium *Lactobacillus plantarum* (Stephenson & Rowatt 1947).

By the 1980s, the term *brain-gut axis* had become a common label for investigations of these connections (e.g., Aziz & Thompson 1998; *Gastroenterology* 1980). A variety of important findings emerged about gut microbes, their cell wall components, and nervous systems or behavioural states (Bluthé et al. 1992; Hart 1988; Lyte 1993). Further extensive experimentation on adrenaline and noradrenaline showed that they stimulate the growth of some bacteria (Lyte & Ernst 1992) and that some microorganisms are themselves able to produce these substances (Asano et al. 2012; Tsavkelova et al. 2000). This body of evidence that microbes can influence the gut-brain axis, and in turn be influenced by the brain-gut axis, forms an important basis for more recent developments in MGB research.

From the microbiological angle, intestinal microbes have long been studied for their effects on human health, from the perspectives of both individual pathogens and more systemic community effects (see Haenel 1961; Savage 2001). Although work in the 1980s had begun to examine mechanistically how specific intestinal microorganisms might affect mammalian brain states (e.g., Brown et al. 1990; Jeppsson et al. 1983), it is only in the last decade that brain-gut-axis research has been able to take advantage of methods that reveal the full diversity of microorganisms inhabiting the human gut. This expanded capacity for the molecular analysis of microbial communities in host organisms is what is now called *microbiome research*.

Microbiome research developed on the basis of tools that allow analysis of the DNA sequence of entire microbial communities (microbiota). The DNA is directly extracted from microbiota in their natural environments (Handelsman et al. 1998; see sect. 5 for more detail). "Microbiomes," the molecular sequences of these communities, are analysed for compositional patterns and their associations with aspects of the environment. In the mid-2000s, microbiome researchers began to focus more closely on the human ecosystem: the human body and its complement of microorganisms, particularly gut microbes (Eckburg et al. 2005). As human microbiome research developed, key researchers began to use germ-free (GF) mice. These are mice that are born and live their lives without microorganisms until they are experimentally colonized; other GF organisms have been used historically for different purposes (Kirk 2012). Influential studies showed that giving GF mice microbiota transplants from obese hosts could bring about obesity (e.g., Turnbaugh et al. 2008). Although GF mice have many abnormalities (see sect. 7.3), they have become the gold experimental standard for causal claims in human microbiome research, which now includes gut-brain studies.

Despite all of these well-known MGB precursors, the current phase of microbiome-oriented gut-brain research often cites its starting point as 2004, when Sudo and colleagues (2004) used germ-free mice to reveal that "commensal microbes [are] affecting the neural network responsible for controlling stress responsiveness" (p. 271). Many of today's microbiota-gut-brain papers refer to the Sudo et al. paper as "seminal" (e.g., Mayer et al. 2015, p. 926; Sampson & Mazmanian 2015, p. 567) and as a "landmark" in the history of the emerging field of MGB research (e.g., Foster & McVey Neufeld 2013, p. 306). This 2004 paper emphasizes a simple potential treatment: probiotics. It also suggests that GF mice allow much of the complexity of microbiomes to be ignored: Mice either have microbiota or they do not. Both this paper and the earlier work have inspired attempts to merge multiple disciplinary perspectives, including those from psychiatry, pharmacology, psychology, neuroscience, immunology, microbiology, and gastroenterology. But in the process of drawing on so many approaches, key problems plaguing broader

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microbiome analyses were also included: the difficulty of identifying causal pathways and yet the tendency to suggest microbiota are bringing about specific host effects (see Hanage 2014).

3. MGB research and its scope

In part because of its rich historical background, MGB studies draw on a considerable variety of methods and disciplinary approaches (see Supplementary Table 1). These methods are both experimental and descriptive. They focus on implementing microbiota-related interventions that can change specified brain and/or behavioural states. The targets of these interventions are usually disorders of various degrees, including depression (Jiang et al. 2015; Park et al. 2013), anxiety (Crumeyrolle-Arias et al. 2014; Neufeld et al. 2011a), autism (de Theije et al. 2014; Hsiao et al. 2013), schizophrenia (Severance et al. 2016), posttraumatic stress disorder (Hemmings et al. 2017), Parkinson's (Sampson et al. 2016), and anorexia nervosa (Kleiman et al. 2015). But more general brain and behavioural states are also scrutinized, including fear (Bravo et al. 2011), stress (O'Mahony et al. 2017), mood (Steenbergen et al. 2015), temperament (Christian et al. 2015), cognition (Magnusson et al. 2015), memory (Gareau et al. 2011), and sociability (Desbonnet et al. 2014).

When experimental effects are detected, mechanisms are often postulated to consolidate the links made between these brain and behavioural outcomes and the microbiota. Proposed intermediary mechanisms include the vagus nerve, inflammatory molecules, microbial metabolites and "neurotransmitters," immune system mediators and responses, various "signalling" molecules and cells, the so-called leaky gut, and leaky blood-brain barriers (see Sampson & Mazmanian 2015). None of these are uncontested as potential or adequate mechanisms. For example, the molecules often labelled "neurotransmitters" are not neurotransmitters for the microbes. Even if these molecules can cross the gut barrier and blood-brain or nerve barriers, they do not meet the criteria for neurotransmitters. These criteria require a neurotransmitter to be present in presynaptic elements, for it to be released in response to presynaptic depolarization and for there to be receptors on a postsynaptic cell (Purves et al. 2001). Another very problematic mechanism is the "leaky gut" and its highly disputed role in neurological disorders (e.g., Quigley 2016; Rao & Gershon 2016; see sect. 7).

An outline of some key studies in MGB research will help show the field's scope and trajectory of development. The now classic Sudo et al. (2004) paper serves as something of a template for much subsequent research. In that paper, Sudo et al. compare hypothalamo-pituitary-axis (HPA) responses to restraint stress in GF, specific pathogen-free (SPF), and conventional mice (i.e., unmanipulated microbiota). The study found that GF mice show higher post-stress corticosterone concentrations than SPF and conventional mice. In addition, higher corticosterone in GF mice was counteracted by the administration of probiotic bacteria (*Bifidobacterium infantis*). Because this occurred only to the 9-week-old mice and not the older ones (17 weeks), Sudo et al. (2004) postulated a crucial developmental stage for the HPA stress response that is determined by microbiota. These key findings of probiotic effects on physiology and behaviour, plus a developmental window of maximum effect, get taken up in numerous other MGB papers.

In 2009, O'Mahony and colleagues established that several consequences of maternal separation stress exist at adulthood: namely, visceral hypersensitivity, changes in gut microbiota, less exploration of novel environments, and more defecation. Those behaviours are often considered "anxiety-like" (see sect. 6 for further discussion). The relationship of such behaviour to microbes had already been explored in earlier work focused on single microbes (e.g., Lyte et al. 1998). Following the new trend of focusing more broadly on the microbiota as a whole, Diaz Heijtz et al. (2011) and Neufeld et al. (2011a) found that GF mice (i.e., no microbiota at all) display fewer anxiety-like behaviours than SPF mice in the light-dark box and elevated plus maze.

In the same year, Bercik et al. (2011a) published findings of the effects of oral antibiotics on anxiety-like behaviour in the step-down and light preference tests. Comparisons were made after microbiota transplantations into SPF Balb/C mice (an inbred mouse strain widely used in immunology and considered to display a high level of anxiety-like behaviour, or "timidity"), National Institutes of Health (NIH) Swiss mice (an outbred strain that shows less anxiety-like behaviour, or greater "boldness"), or GF Balb/C mice. The study found that oral antibiotic treatment reduced anxiety-like behaviour and increased exploration of the behavioural devices used, and that this increased exploration did not involve autonomic nerves. In addition, Bercik et al. (2011a) reported that Balb/C recipient mice transplanted with NIH Swiss microbiota showed more exploration than their counterparts with only Balb/C microbiota. Conversely, NIH Swiss mice that received Balb/C microbiota transplantation displayed less exploration than those that were colonized with NIH Swiss microbiota. The success of these interventions suggested to many people in the field that the microbiota is a major causal agent in determining anxiety-like behaviour.

Making a narrower microbial intervention (i.e., just one microbe, not a community), Bravo et al. (2011) used a probiotic bacterium (*Lactobacillus*) to manipulate anxiety-like and depression-related behaviours in mice. They examined depression-related behaviour with the forced swim test (measuring how long the animal was immobile) and anxiety-related behaviour by the number of entries on to the open arms of the elevated plus maze. They also measured the time spent freezing after fear conditioning with a mild electric shock. Probiotic administration reduced immobility during forced swim tests and increased the number of open arm entries in the elevated maze. Subdiaphragmatic vagotomy (severing the vagus nerve under the diaphragm) prevented these effects (however, see Bercik et al. [2011a], who found no role for the vagus nerve in modulating the effects of antibiotics on the behaviour of mice in the light-dark preference and step-down tests). Follow-up studies subsequently showed that the probiotic facilitates firing of vagal sensory fibres (Perez-Burgos et al. 2013). Findings such as these have given rise to the idea of "psychobiotics." These are substances derived from microorganisms that can be used as treatments for improving mental health (Dinan et al. 2013). This notion has strong appeal inside and outside of the MGB field.

A study by Hsaio et al. (2013) suggested how such interventions might work mechanistically. The authors used adult mice born from mothers that had been administered an immune stimulation (a viral mimic) during pregnancy. The pups were born with both a "leaky gut" and the behavioural features of autistic developmental disorders. The adult offspring displayed anxiety-like features in the open field, stereotypical behaviour, less social interaction, and fewer ultrasound vocalizations. Feeding *Bacteroides fragilis* to the impaired mice mitigated "obsessive" behaviours such as grooming and marble-burying. However, reduced sociability did not improve, which was attributed to

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developmental timing. *B. fragilis* was known from earlier immunological studies to improve immune defects (Mazmanian et al. 2008). Although Hsaio et al. did not isolate colonized *B. fragilis* in the mouse intestines, a metabolic mediator associated with this microorganism was restored to normal levels after probiotic treatment. Studies such as this, although still incomplete, hint at the potential mechanistic pathways that might underlie microbiota effects on brain and behaviour.

Many MGB studies, including those just discussed, are believed to be relevant to human psychiatric disorders. In addition, cognitive and behavioural processes that are not necessarily connected to any psychiatric disorder have also been linked to microbiota changes. Bravo et al. (2011) showed that although no differences in the amount of behavioural freezing were observed immediately after mice received a foot shock, mice that were fed a probiotic showed more conditioned freezing the next day than probiotic-free mice. Diet also has effects. Non-obese antibiotic-pretreated mice were given microbiota transplants from animals fed a high-fat diet. The mice with the high-fat microbiota transplants displayed more conditioned freezing to a shock-signalling tone than did mice with transplants from animals on a control diet (Bruce-Keller et al. 2015). Gareau et al. (2011) observed that probiotics could reverse stress-induced deficits in novel object recognition. Antibiotic treatment of healthy mice from adolescence through adulthood was also found to impair novel object recognition in mice (Desbonnet et al. 2015).

Whether about cognitive or emotional capacities, or aspects of psychiatric disorders, the potential implications of these and many other studies are striking. Many of the core findings and interpretations are echoed repeatedly in the general MGB literature, which is characterized by an abundance of reviews (see sect. 1 in the Supplementary Material). Some of this work then goes so far as to claim that microbes control the mind and that free will is thereby refuted (e.g., Lepage et al. 2013; Stilling et al. 2016; see sect. 7). Most of these reviews, as well as much primary research, proclaim that a conceptual and methodological revolution is underway in brain and behavioural research (e.g., Liu 2017; Mayer et al. 2014). And yet much of the research is highly speculative regarding causation and mechanisms, some of it is contradictory, and many well-established methods are used in limited, mistaken, and even outdated ways, as we will show.

Although some scientific papers and popular essays have already pointed toward central problems for microbiome research (e.g., Eisen 2017; Shanahan & Quigley 2014) and warnings have been issued about MGB "hype" in particular (see comments in Smith 2015; Zimmer 2014), these discussions have not been based on detailed examinations of the core literature. Very commonly within the field, cautionary statements are embedded in strongly promotional overviews of MGB research (e.g., Mayer et al. 2014; Sherwin et al. 2018). Our aim is to provide a more thorough critical and external analysis of the field for anyone who wants to understand human minds and behaviours, and their putative microbiome connections.

4. The 25 most cited MGB papers

In order to analyse the field more closely, we examined the most highly cited MGB papers in the last decade (Table 1). We chose this set of papers because of their importance to the established field, and particularly its experimental core. They have shaped the field and continue to structure it, as all their citations attest. Focusing on them allows us to probe deeply into influential methods and interpretations, which would be less effectively achieved in a comprehensive but relatively shallow overview of all existing literature. Although we recognize that this selection of papers will not include the most recent work in the field (some of which may be using improved techniques), our aim here is to capture the most recognized experimental work that has been the basis for the majority of reviews and subsequent studies, as well as media attention.

To identify this central corpus of work, we carried out a PubMed search using the term "gut-brain microbiota" (date of access: May 25, 2017; updated July 11, 2018). We discarded all reviews, which formed a very high proportion of the literature (almost 50%; see Supplementary Material). This search found 325 articles. We then used Google Scholar citation counts for each article to rank all the papers with more than 150 citations (a total of 15). To supplement this core of highly cited papers, we also examined the references to open access articles within the original 325 articles. This strategy found another 9 highly cited articles. Finally, we conducted a third search using the looser term "brain microbiota." This search found 867 articles. We inspected the most cited articles of this group, which revealed another 3 publications that had not appeared in our earlier "gut-brain microbiota" search. We slightly cropped this list to 25 papers, of which the lowest number of citations is just over 120 and the highest above 1300 (Table 1). We then analysed the text of these papers manually, with an initial focus on two categories of methodology: microbiome methods (sect. 5) and behavioural tests and statistics (sect. 6).

5. Microbiome methodology

Microbiome research relies on the rapid and extensive DNA profiling of bacterial and other microorganismal genomes in specified locations. This use of DNA sequencing tools to explore microbial biodiversity is often called *metagenomics*, meaning that it goes beyond the single-species genome analyses of genomics (Handelsman 2004). It allows the investigation of microbial communities in a vast variety of environments, including those provided by animal hosts. These methods have liberated the study of microbial biodiversity from the constraints of pure culture. Pure culturing approaches require growing microorganisms in the laboratory, which is not feasible (yet) for many microorganismal groups.

In the simplest scenario for sequencing, the presence of species is evaluated with metagenomic methods, which can be performed in two ways. The first is tag (or amplicon) sequencing, usually of a particular stretch of a ribosomal gene. The second is shotgun sequencing, which captures all the genes in the environmental sample. Tag sequencing is still widely used despite being restricted to information about bacterial abundance and diversity. Shotgun sequencing provides more information about the total pool of genes present in the environment but requires more complicated bioinformatic analysis. In order to do more than catalogue taxa on the basis of genes, researchers also employ metatranscriptomic methods to find actively transcribed genes, and metabolomic analyses to quantify the output of bacterial metabolic pathways (see Knight et al. [2018] for an updated methodological primer). However, whether tag or shotgun methods are used, the bulk of microbiome research has yet to advance beyond gene catalogues, and this greatly limits what can be said about microbial effects on hosts and other environments. But as we will show, a surprising

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Table 1. The 25 most cited papers in MGB research[a]

Publication	Citations
Diaz Heijtz et al. (2011) Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences of the United States of America*	1,348
Bravo et al. (2011) Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences of the United States of America*	1,218
Hsiao et al. (2013) Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell*	1,173
Sudo et al. (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *Journal of Physiology*	935
Bercik et al. (2011a) The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology*	701
O'Mahony et al. (2009) Early life stress alters behavior, immunity, and microbiota in rats: Implications for irritable bowel syndrome and psychiatric illnesses. *Biological Psychiatry*	613
Neufeld et al. (2011b) Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology and Motility*	613
Tillisch et al. (2013) Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology*	596
Messaoudi et al. (2011) Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. *British Journal of Nutrition*	594
Clarke et al. (2013) The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Molecular Psychiatry*	507
Bailey et al. (2011) Exposure to a social stressor alters the structure of the intestinal microbiota: Implications for stressor-induced immunomodulation. *Brain, Behavior, and Immunity*	467
Gareau et al. (2011) Bacterial infection causes stress-induced memory dysfunction in mice. *Gut*	351
Jiang et al. (2015) Altered fecal microbiota composition in patients with major depressive disorder. *Brain, Behavior, and Immunity*	260
Ait-Belgnaoui et al. (2012) Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology*	234
Steenbergen et al. (2015) A randomized controlled trial to test the effect of multispecies probiotics on cognitive reactivity to sad mood. *Brain, Behavior, and Immunity*	211
Leclercq et al. (2014) Intestinal permeability, gut-bacterial dysbiosis, and behavioral markers of alcohol-dependence severity. *Proceedings of the National Academy of Sciences of the United States of America*	185
Bajaj et al. (2013) Modulation of the metabiome by rifaximin in patients with cirrhosis and minimal hepatic encephalopathy. *PLoS ONE*	176
Crumeyrolle-Arias et al. (2014) Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. *Psychoneuroendocrinology*	173
De Theije et al. (2014) Altered gut microbiota and activity in a murine model of autism spectrum disorders. *Brain, Behavior, and Immunity*	166

(*Continued*)

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Table 1. (*Continued.*)

Publication	Citations
Bruce-Keller et al. (2015) Obese-type gut microbiota induce neurobehavioral changes in the absence of obesity. *Biological Psychiatry*	161
Desbonnet et al. (2015) Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain, Behavior, and Immunity*	159
Neufeld et al. (2011a) Effects of intestinal microbiota on anxiety-like behavior. *Communicative & Integrative Biology*	145
Ohland et al. (2013) Effects of *Lactobacillus helveticus* on murine behavior are dependent on diet and genotype and correlate with alterations in the gut microbiome. *Psychoneuroendocrinology*	135
Ait-Belgnaoui et al. (2014) Probiotic gut effect prevents the chronic psychological stress-induced brain activity abnormality in mice. *Neurogastroenterology and Motility*	131
Park et al. (2013) Altered colonic function and microbiota profile in a mouse model of chronic depression. *Neurogastroenterology and Motility*	130

[a]Papers were extracted using a combination of PubMed searches and Google Scholar citations. See the main text for detailed selection methods. Papers are ranked by the number of citations received.

amount of the MGB research in our top-cited sample does not even achieve the cataloguing step.

The gut is home to the most studied but also the most complex human-associated microbiota. It contains hundreds if not thousands of different microbial species, of which bacteria are the main component and research focus. The relative abundance and diversity of bacteria can vary considerably from one individual human to another (Human Microbiome Project Consortium 2012). Difficulties in interpreting diverse and complex sequence data result in the main output of health-focused microbiome studies being simple correlations between the abundance of particular taxa and host-associated disease states. These association patterns do not allow cause and effect to be ascertained (de Vos & de Vos 2012; Hanage 2014). Moreover, the great majority of investigation is done with faecal samples, which are unlikely to represent microbial activity in the gut itself, especially in the small intestine or in association with the mucosal surface (Gevers et al. 2014; Momozawa et al. 2011; Quigley 2017). Nevertheless, the sheer convenience of such samples continues to ensure their popularity.

How does microbiome research feature in MGB studies? In general, most MGB papers are not microbiome-driven in the way many other health-related or environmental microbiome papers are. In fact, in MGB research, including our 25 most cited list, "microbiota" and "microbiome" are often used simply to indicate that microorganisms in the human body appear to be involved in producing observed effects. Despite many methodological advances in microbiome research, standard microbiome analyses are not carried out even in many of the most highly cited MGB papers.

There are four broad categories of "microbiota" methods in the 25 most cited MGB papers we analysed.

1. Comparisons of behaviours in GF mice/rat microbiomes with conventionally colonized or SPF animals (e.g., Crumeyrolle-Arias et al. 2014; Gareau et al. 2011; Sudo et al. 2004). Sometimes a rescue experiment is performed in which a standard microbiota is transplanted into GF animals to investigate whether the phenotype can be reversed (Clarke et al. 2013; Diaz Heijtz et al. 2011; Neufeld et al. 2011a; 2011b).
2. Studies of normally colonized mice treated with antibiotics (Ait-Belgnaoui et al. 2012; Bajaj et al. 2013; Bercik et al. 2011a; Desbonnet et al. 2015). One study in our sample then re-colonized the animals with microbiota from obese and normal hosts (Bruce-Keller et al. 2015).
3. Studies in which probiotics and placebos are given to human or other animal subjects (Supplementary Table 2).
4. Standard microbiota studies that assess the experimental alteration of gut microbes (Supplementary Table 3). Some older methods are still used to describe the microbial community, such as denaturing gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP). But at least some MGB researchers are now turning to more contemporary methods such as quantitative polymerase chain reaction (qPCR), which is an amplification method that targets specific molecules and thus selected taxa, or shotgun DNA sequencing that encompasses the whole community.

For most of the interventions in the third category of "microbiota" methods (probiotics), *Bifidobacterium* sp. and *Lactobacillus* sp. are the probiotics of choice, with *Lactobacillus helveticus* being the most popular (Supplementary Table 2). These genera of organisms have long been traditional targets for claims about fermented milk products having digestive and physiological benefits (e.g., Metchnikoff 1908). *B. fragilis*, the intervention microorganism in Hsiao et al.'s (2013) study, is not found in fermented milk products but can be deployed according to the World Health Organization (WHO) definition of a probiotic: any live microorganism that is used to intervene in a human body to bring about health effects (Hill et al. 2014; however, see Shanahan & Quigley [2014] for conceptual concerns). We will come back to probiotics and their implications in section 7.

An important observation to make here is that treatment with single or multiple probiotics is not strictly a "microbiota" or

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"microbiome" study. Normally, this term is reserved for studies in which microbiota samples are analysed bioinformatically after sequencing. In MGB probiotic research, however, researchers might not even profile changes in bacterial composition, and when they do, no differences may be observed (e.g., Tillisch et al. 2013). Surprisingly, even when microbiota *are* analysed for changes, very limited microbiome methodology is used (Supplementary Table 3). The methods that are employed are often not state-of-the-art. It is curious indeed to see much older qualitative methods, such as DGGE, being used for a publication in 2013 (Park et al. 2013). Although a useful tool in the 1990s, community fingerprinting methods like DGGE and T-RFLP have long been superseded by more advanced quantitative sequencing methods. These newer methods allow closer analysis of the composition and potential function of microbial communities.

It is important to note, however, that microbiome research in general continues to have a "causality problem" despite improved sequence analysis tools (Hanage 2014). Many microbiome studies simply cannot isolate specific causes no matter how sophisticated their sequencing and bioinformatic tools; even the experimental work with microbiota transplants is not adequate to demonstrate whole-microbiome causality (O'Malley & Skillings 2018; see sect. 7). In this regard, MGB studies may have an advantage, in that they focus on single microorganisms (probiotics) or small groups of microbes that can be manipulated. However, a probiotic focus would not normally license claims about the whole microbiome, and even narrow probiotic causal claims are problematic (see sect. 7).

A standard interpretation in MGB research is to attribute differences in behaviour between GF and non-GF animals to the lack of microbiota in the former (ditto for antibiotic interventions, which deplete but do not fully remove the microbiota). Often the different treatments experienced by GF or antibiotic-treated mice are not remarked on. Few studies in our most cited sample provide controls that would enable singling out the effects of the microbiota itself (e.g., rescue of phenotype by re-infecting GF animals with a full community transplant or by reintroducing specific bacteria). Although GF models have yielded many interesting results, questions continue to be asked about how relevant they are to humans (Nguyen et al. 2015), because very few humans ever experience germ-free conditions. Although sometimes GF status is equated with environments that have high levels of hygiene and multiple antibiotic treatments (e.g., neonatal care facilities; see Clarke et al. 2013), for the majority of researchers these are not considered equivalent conditions at all.

Overall, there are very few studies in this highly cited group of papers that have an experimental approach genuinely able to demonstrate the impact of the microbiota itself on behaviour. Correlations are loosely interpreted as indications of potential mechanisms (however, see Bajaj et al. [2013] for a more sophisticated analysis of correlation networks of microbial metabolites). The conditions under which potential mechanisms might operate are not specified. For example, one study postulates "the existence of a gut–brain axis in alcohol dependence, in which the gut microbiota could alter the gut-barrier function and influence behavior in alcohol dependence" (Leclercq et al. 2014, p. E4491). Yet all that this particular piece of research demonstrates is a correlation between increased intestinal permeability and certain bacterial taxa. Less cited and newer studies may be making greater efforts to show microbiota causality of behaviour and brain function (see sect. 8), but, in general, invoking the whole microbiome, rather than specific members of it, will require methods that are carefully designed to deal with the complexities of thousands of interacting organisms and pathways.

One consequence of this complexity is that inter-individual variability between human microbiomes is so high that it is impossible – given most clinical sampling practices – to distinguish specific groups of patients or animals and to find the taxa most associated with different health states (e.g., Falony et al. 2016). Frequently, when differences in bacterial composition are observed in the broader body of MGB literature, they are simple correlations from single studies rather than multiple comparative analyses. Considering that hundreds of taxa are involved in any gut community, it is not surprising that some correlations are found. The broader microbiome field (outside MGB) uses a range of statistical correction measures, and their implementation – although still imperfect – at least reduces gross false discovery rates (Knight et al. 2018; Weiss et al. 2016). For example, one of the reasons that standard parametric tests are not adapted to microbiome data is because of the issue of compositionality. Rapid changes to any single taxon in the microbiota are often measured as changes to all of the taxa, instead of reflecting true abundances. This property leads to extremely high false discovery rates. These ongoing issues add to the field's struggles to achieve causal explanations of phenomena such as disease, but their incidence in MGB research is exacerbated by weaknesses in the methods that are used in combination with microbiome analyses.

6. Neuroendocrine, behavioural, and statistical tests

Microbiome research in its standard sense (i.e., the sequencing and bioinformatic analysis of community genomes) might inform only a subset of MGB papers, and even when it is carried out, it is unlikely to be the methodological focus. Most of the methodology is in fact centred on rodent hormones and behaviour in different conditions. We divided the 25 most cited MGB papers into five categories according to their research focus relative to hormones and behaviour: (1) neuroendocrine "stress" axis; (2) emotion-mood: anxiety; (3) mood disorder: depression; (4) autism spectrum/developmental disorders; and (5) cognition (see Supplementary Tables 4a–4e). About half of the 25 top-cited papers are concerned with activation of the so-called neuroendocrine "stress" axis, which results in the production of stress-related glucocorticoid hormones (Supplementary Table 4a). All of these studies, save one, describe experimental work done in rodents. Sixteen of the top 25 papers explore anxiety, of which 13 studies were carried out on rodents (Supplementary Table 4b). A little less than a quarter (6) of the articles are related to depression, with the majority of that work being done in humans (Supplementary Table 4c). Only two studies present work on animal models of autism spectrum disorder (Supplementary Table 4d), and six address different forms of cognition (Supplementary Table 4e).

Most of the studies we examined do not explicitly justify their methodologies. They seldom address potentially confounding effects (e.g., maternal separation and water avoidance stress) that may complicate interpretation and limit the generalizability of findings. The adequacy of particular behavioural tests and measures is rarely discussed and seems to be taken for granted (admittedly because many other studies have done so). For example, following Sudo et al.'s initial 2004 work, about half of the papers in our top-cited sample measure corticosterone in relation to gut microbiota in rodents. Although most of this subset of papers examines corticosterone in the context of stress – a framework

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laid down by formative research published 60 years ago (Eik-Nes & Samuels 1958; Gold et al. 1958; Persky et al. 1958) – it is worth recalling that non-stressful events, such as meal consumption, also increase the circulating concentration of this glucocorticoid (Toda et al. 2004; Wang et al. 1999). Adrenalin can equally be considered a stress hormone (Mormède et al. 2007). In other words, there can be confounding factors at play in any observation of stress responses.

The appropriateness of animal models for human disease is seldom argued for and yet is of crucial importance for the implications of these studies. Not only do mice and humans have different gut structure and neuroanatomy, different microbiota, and different evolved behaviours (see Arrieta et al. 2016; Nguyen et al. 2015), but there are also acute problems of "translation" into clinical practice when it comes to claims about stress, anxiety, and depression. Behaviours that may be normal for mice (e.g., fearfulness and timidity) are not normal or desirable for humans, and vice versa. Moreover, no self-report-based evaluations can be made on rodents to gain better insight into the organism's experience. Although terminology about findings related to disorders is generally appropriate in the 25 papers we examined most closely (e.g., "anxiety-like" and "depression-like"), we nevertheless found several instances of terms for multidimensional human disorders (e.g., "anxiety" and "depression") being applied to the unidimensional rodent results (see Supplementary Tables 5a, 5b).

Translational issues arise in any research that extrapolates from rodent models to human function (Zeiss & Johnson 2017) but are particularly pertinent to neuropsychiatric disorders (Homberg 2013). In rodent behavioural studies, interpretations of results obtained in the open field, elevated plus maze, light-dark box, and forced swim tests have frequently been criticized. Indeed, some critical reviews recommend finding new animal paradigms to investigate anxiety (Belzung & Griebel 2001). Some authors go so far as to say that "evidence in support of the validity of the plus-maze, the light/dark box and the open-field as anxiety tests is poor and methodologically questionable" (Ennaceur 2014, p. 55). Other authors consider increased immobility in the forced swim test an adaptive passive coping strategy rather than a measure of the behavioural despair that is indicative of human depression-like behaviour (Commons et al. 2017; Molendijk & de Kloet 2015).

When articles from our 25 most cited papers *do* take notice of translational issues, they may not take them seriously. For example, Hsiao et al. (2013, p. 1456) quote Bourin et al. (2007) as saying that "mapping an animal's movement in an open arena" allows researchers "to measure … anxiety." Crucially, however, Bourin and colleagues are arguing that it is important to specify whether the open field test is used under dimly lit conditions to measure mere locomotor activity, or whether implementing it in bright light is testing innate rodent anxiety of open spaces during the day. Bourin et al. (contra Hsiao et al.'s interpretation) go on to urge caution about interpreting findings as having implications for anxiety disorders (Bourin et al. 2007). In the broader MGB field (i.e., beyond the top-cited papers), there are some examples of researchers supplementing or changing their reliance on the open field and elevated maze plus tests (e.g., Bassi et al. 2012; Goehler et al. 2008), in order to avoid the confounding of anxiety-like behaviour with simple alterations in locomotor activity patterns (Swiergiel & Dunn 2007). Most commonly, however, if mentioning these issues, MGB researchers merely note them and then very pragmatically continue with animal model manipulations and interpretations.

To conclude our methodological analysis, there are reasons to think that the statistical analyses carried out by some MGB studies in our most cited sample are not appropriate (see Supplementary Table 6). In particular, one-way analyses of variance (ANOVAs) or Student's *t*-tests are frequently employed when the experimental design includes more than one independent variable. In such cases (e.g., Ait-Belgnaoui et al. 2012; 2014; Ohland et al. 2013), two- or three-way ANOVAs are required. In many biological situations, the effect of one factor on an outcome of interest often depends on other factors. Hence, when two or more independent variables or factors (such as microbiota status and stress) are studied, it is important to address both the effects of those factors independently and their interaction with the dependent variable being measured (e.g., behaviour in a specific test). Several of the 25 most cited papers did not do this (Supplementary Table 6). Finally, in a few of the MGB papers we analysed, statistically negative results ($P > 0.10$) are presented as if they are positive findings. For example, non-significant findings after intervention strategies on the microbiota are still used to argue for potential microbiome effects (see Bailey et al. 2011; Bravo et al. 2011; Tillisch et al. 2013). It would be much more straightforward to say "no effect is found" without assuming other methods or future experiments on larger cohorts will find the desired outcomes.

Following Fisher, it is standard in the life sciences to consider $P < 0.05$ as statistically significant and, conversely, that $P > 0.05$ indicates a non-significant difference (Habibzadeh 2013). In this context, it is not possible to talk about "marginally significant" or "partially significant" (Habibzadeh 2013), or as noted previously, "potentially significant." At best, a statistical trend can be inferred when $0.10 < P < 0.05$, provided there is sufficient statistical power. But if anything, studies in the life sciences tend to be underpowered, which has led several authors to make a plea for the use of more stringent cut-offs for P values and to consider only $P < 0.01$ as statistically significant (e.g., Colquhoun 2014; Vidgen & Yasseri 2016). MGB research has yet to reflect on this advice.

These behavioural and statistical testing problems are by no means exclusive to MGB research. In fact, they are common throughout rodent-based behavioural neuroscience (Button et al. 2013). But in MGB research, these weaknesses are compounded by the fact that it is misleading in some of the papers even to refer to microbiomes because no such analysis is done. Even when it is, superseded methods are providing very low-quality analyses. It is difficult of course to do everything well in interdisciplinary research, but, in some instances, it seems as if MGB papers are simply invoking the term "microbiome" without appreciating the minimal methodological commitments with which the term is normally accompanied.

7. Strong claims and interpretations

Although many of our 25 most cited papers use fairly basic reasoning, with limited mechanistic detail, they do not by and large indulge in the overinterpretation and overstatement to the extent we found in some of the broader MGB research literature. However, both our smaller sample of top-cited papers and the larger body of literature we examined divulge many examples of papers in which strong claims – such as "conclusively demonstrate" and "conclusive proof" (e.g., Ait-Belgnaoui et al. 2014; Bravo et al. 2011) – are offset by more conservative elaborations, sometimes in the very same paper (e.g., Christian et al. 2015;

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Foster & McVey Neufeld 2013). We are tempted to diagnose this as a case of "double-dipping," when cautionary statements are belied by much more dramatic claims. We believe this strategy influences the public uptake of MGB research. In the following sub-sections, we discuss a selection of the overblown conclusions or speculations that help inflame the field, from the most abstract to the highly practical. We do this in order to show how misinterpretation may arise and propagate, especially in the review papers that are so dominant in MGB literature (between 40% and 50%; see sect. 1 in the Supplementary Material).

7.1. Claims about causality and determinism

In the wider field of health-related microbiome research, there are many recognized difficulties in extracting cause-effect relationships from microbiome data (e.g., Hanage 2014; Surana & Kasper 2017), largely because of how the standard methodology works. Microbiome analysis is basically descriptive, not explanatory. Many efforts are currently underway to explore and assess causal claims, but these attempts are hampered by the whole-community focus of much microbiome methodology. Because microbiome methods begin with communities, there are often expectations that explanations will be found at the community level too, rather than at the level of populations of individual organisms and specific biochemical pathways (e.g., O'Malley & Skillings 2018; Rosen & Palm 2017).

We can see this problem most clearly when MGB researchers attribute changes in human health to changes in the community of gut microorganisms. These changes can be simple shifts in the relative proportions of groups of microorganisms in the community (e.g., Bailey et al. 2011; Jiang et al. 2015) or in reference to "normal" community compositions (Clarke et al. 2013; Leclercq et al. 2014). One of our top-25 articles attributed memory-regulating causality to the mere presence of a microbiota, rather than any particular composition (Gareau et al. 2011), as did Sudo et al. (2004) for stress response. This is a general message gleaned from GF mouse studies, where the causal variable can be the simple presence or absence of a microbiota. In other papers, community-level differences are often assigned causal roles under the banner of "dysbiosis."

Dysbiosis is frequently defined as either a broad change or an "imbalance" in microbiota that produces a diseased state in the (human) host (e.g., Mazmanian et al. 2008). Many of our 25 most cited papers adopt this loose definition (e.g., Bercik et al. 2011a; Hsiao et al. 2013; Leclercq et al. 2014), and the term circulates widely in the MGB literature. However, considering the extensive inter-individual variation between each human microbiome, it is very difficult to define what constitutes a "normal" or "healthy" or "balanced" microbiome (Hooks & O'Malley 2017). With such a loose definition, dysbiosis can mean any change in microbiota between two compared groups of patients or animals. Even assumptions that "reduced diversity" is linked to illness outcomes (e.g., Desbonnet et al. 2015) are problematic, because some disease states are associated with increased diversity (Shade 2017; Zaneveld et al. 2017).

Worryingly, one of our 25 most cited papers postulated a role for dysbiosis even when no compositional microbiome differences were found pre- and post-intervention in healthy humans (Tillisch et al. 2013). Many papers discussing dysbiosis go on to assume that when microbiome changes and illness co-occur, the causal pathway will be from microbiota to the disease state rather than the other way round, or from another common cause (e.g., Bruce-Keller et al. 2015; Crumeyrolle-Arias et al. 2014; O'Mahony et al. 2009). However, some MGB papers are now taking more nuanced perspectives on dysbiosis "causality" (e.g., Ohland et al. 2013; Park et al. 2013), and the concept is currently receiving considerable critical attention and retheorizing in the broader microbiome literature (e.g., Hooks & O'Malley 2017; Olesen & Alm 2016; Shanahan & Quigley 2014; Zaneveld et al. 2017).

Lying behind the whole-community causation issue is an even stronger one, of microbiota "determinism." By this we mean bold claims that are made about human dependency on microbes for many aspects of health (e.g., metabolic, immune, and neuroendocrine systems – see Bercik et al. 2011a; Neufeld et al. 2011a). These claims include mental health, to the extent that some MGB review papers even suggest our microbiota "control" and "manipulate" our brains (e.g., Stilling et al. 2016) or "hijack" our central nervous system (e.g., Alcock et al. 2014). The ability of microbes to determine what we often consider to be central nervous system capacities and states (mood, cognition, emotion, etc.) is a radical one and is probably employed more for provocation than serious consideration. Almost all MGB papers recognize in their small print the lack of a causal account of how microbiota changes are connected to brain and behavioural states. And yet underlying dramatic suggestions that MGB research does away with free will conceptions (e.g., Lepage et al. 2013) is a more reasoned position that microbes are "benevolent" manipulators, and that evolution has made them so. Can evolutionary theory back up such claims?

7.2. Claims about the evolved benefits of microbiota for brain states

There are numerous MGB articles (including some within the 25 most cited sample) that suggest we have a beneficial relationship with many if not all of our microbiota (e.g., Bailey et al. 2011; Sudo et al. 2004). The reason for this, according to at least some MGB researchers in the broader literature, is supposedly that our long evolutionary association with microorganisms has eradicated conflict (e.g., Stilling et al. 2016). In other words, natural selection has selected against competitive relationships in the history of human evolution, and we should therefore find the evolved ways in which to maintain the right "balance" with our microbiota (e.g., Wang & Kasper 2014).

Many such MGB claims begin with the central example of *Toxoplasma gondii* as a single organism capable of having manipulative effects on animal brains and behaviour (e.g., Mayer et al. 2014; Sampson & Mazmanian 2015; Stilling et al. 2016). *Toxoplasma* is a single pathogen, and therefore neither benevolent nor a community, but MGB researchers use it to provide an explanatory template for how microbes manipulate. In the classic account of this parasite's effects, *Toxoplasma* has evolved to infect cats via rodents, and so the former "manipulates" rodent brains in order to make rodents more likely to be consumed by cats (e.g., Berdoy et al. 2000). Changed rodent behaviours include attraction to cat urine and odour. However, there are recognized problems in seeing *Toxoplasma* as evolved by adaptation to change mouse behaviour (Worth et al. 2013). More generally, "microbial manipulation" of any host is better explained as a by-product of the interactions between competing microorganisms in the gut environment (Johnson & Foster 2018). In other words, "manipulation" is a considerable overinterpretation of what the microorganisms are doing and how they have their effects.

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But what about the generally beneficial nature of microbiota? Some MGB and other microbiome researchers have argued that a long evolutionary association between humans and their microbiota has led to benefits and no conflict (e.g., Stilling et al. 2016). Evolutionary theory does not support such beliefs. Communities can be stable and perpetuated over evolutionary time with strongly competitive interactions between different microorganismal populations, and between a human host and the whole microbial community (Coyte et al. 2015). Humans are most parsimoniously understood as an environment for microorganisms, and there are mechanisms of human control and selection over inevitable microbial occupants (Schluter & Foster 2012). There can be negative or positive interactions, as well as neutral ones, and at the moment, microbiome research is unable to separate them out (though efforts are being made to identify key individual microorganisms for specific diseases). But just as for dysbiosis, thinking of whole communities as bringing about specific brain and behavioural (or other physiological) states is very difficult to justify, even (or perhaps especially) within the embrace of evolutionary reasoning.

7.3. Claims about coevolved developmental impact and critical windows

The "coevolved" nature of developmental programmes and microbiota is also argued by the MGB community, both in the 25 papers we examined most closely and more broadly (e.g., Diaz Heijtz et al. 2011; Stilling et al. 2014). Usually, these mentions of "coevolution" do not employ the term in the same way as evolutionary biologists, for whom coevolution means selected reciprocal genetic changes that have been explicitly identified (e.g., Moran & Sloan 2015). In MGB research, coevolution simply means it appears as if the organisms have some evolutionary history together. Even in this very loose sense, there are problems. For example, the effects of colonizing GF mouse pups (Diaz Heijtz et al. 2011) and of probiotic treatments on a maternal infection autism mouse model (Hsiao et al. 2013) have contributed to interpretations of "coevolution" producing a critical timing point for microbial participation in host gut and brain development. However, interpretations of a critical developmental period for microbiome colonization clash with other findings showing that the microbial colonization of GF adult rodents brings about the same effects as it does for much younger GF animals (Nishino et al. 2013). Findings that only male mice are affected developmentally by microbial manipulations are also problematic for general proposals of species-wide neurodevelopmental roles for microbiota (Clarke et al. 2013).

There may also be alternative explanations for apparent critical windows of microbiota effects in animal development. The consequences of manipulating gut microbiota on the physiology and behaviour of an organism may be attributable to more traditionally conceived developmental effects. For example, it is has been shown that GF animals have a more permeable blood-brain interface and larger, but less metabolically active enteric neurons during pre- and postnatal development (Braniste et al. 2014; Dupont et al. 1965). Given that the enteric nervous system and the blood-brain barrier are essential for the normal functioning of gut and brain, it would not, therefore, be surprising to observe atypical behaviour in an adult animal with abnormal development of these systems. However, any behavioural changes do not imply that gut microbiota "control" or "drive" a particular behaviour, but merely that the presence of microbes in the gut may constitute environmental signals to which the developing animal responds by putting in place an enteric neuronal network and a blood-brain barrier.

The adoption of evolutionary-developmental (evo-devo) frameworks in MGB research has also led to studies intimating that if microbes have a big effect on brain development, this must also be occurring prenatally. Some MGB researchers hint that there are large numbers of microorganisms in utero, and that these organisms are having a pre-birth impact on the foetal brain (e.g., Borre et al. 2014; O'Mahony et al. 2017). Yet if they are, current orthodoxy of a mostly sterile pre-birth state would have to be revised.

Recent analysis casts considerable doubt on the potential for in utero colonization and concludes that apparent findings of such colonization are artefactual (Perez-Muñoz et al. 2017). Low-microbial biomass samples, such as those extracted from placenta, yield a similar composition to those from negative controls and are, in fact, dependent on the type or even batch of the kit used to extract and examine the DNA sample. This is the so-called kit-ome problem (see Kim et al. 2017a). Artefacts such as these can be more straightforward explanations of what are otherwise very surprising microbiome findings. That said, we have no doubt that *something* is going on in an evo-devo sense with microbiota and brains. But expecting simple and straightforward findings and linear causal accounts of these interactions does not seem to us realistic, given existing knowledge and methodological sophistication in standard developmental research. There are other oversimplified causal stories that MGB research needs to confront, and chief amongst them are claims about probiotics.

7.4. Probiotic issues

Using the template of the Sudo et al. (2004) study, many subsequent MGB projects (including those in the 25 most cited papers) have made interventions with probiotics on mice and humans and claimed that probiotic interactions with indigenous microbiota affect physiology and behaviour (e.g., Diaz Heijtz et al. 2011; Lyte 2011; Messaoudi et al. 2011; Slykerman et al. 2017; Steenbergen et al. 2015; see Table 1). Often this interaction is conceptualized as the abnormal or "dysbiotic" microbiota being "normalized" by the probiotic (e.g., Ait-Belgnaoui et al. 2012). However, probiotics are a much contested form of intervention. Meta-analyses are equivocal at best about probiotics having positive effects on healthy humans, and their impact is documented for only a few specific disease states (Huang et al. 2016; McKean et al. 2017). At least two randomized controlled trials have found no human effects from probiotic bacteria on human mood or mental health (Kelly et al. 2017; Romijn et al. 2017), whereas a recent meta-analysis (Ng et al. 2017) observed no general mood improvement after using probiotics and only a small effect in patients with mild to moderate depressive symptoms. Concerns have also been raised about the potentially negative alteration of microbiota by probiotics (Slashinski et al. 2012). However, mouse studies do seem to show probiotics having consistent effects on behaviour (Wang et al. 2016), and such findings continue to galvanize the MGB field.

Even if probiotics do have positive effects on guts and brains, some studies show this may not be happening through alterations of the microbiome composition (Kristensen et al. 2016; McNulty et al. 2011). Recent work shows that probiotics do not reliably colonize mouse guts, and do so only to a limited extent for humans (Zmora et al. 2018). Sampson and Mazmanian (2015) account for

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such complications by suggesting more indirect causal routes: "behavioral and neurological changes may not necessarily be a direct function of the specific species of bacteria within the probiotic treatment; rather, microbial-mediated effects on emotion may be due to broader functionality of the community of symbiotic bacteria in the gut" (p. 568). Claims like these fall into what we call the whole-system causation problem that is central to the "dysbiosis" problem (see sect. 7.1). They are very difficult claims to test, especially in a medical context. One of the most cited MGB papers, Ohland et al. (2013, p. 1746), carefully concludes:

> It is clear that diet and probiotics interact at several different levels to alter host physiology. It is likely that not only do the existing gut microbes of the host alter functionality of any given probiotic, but also the diet of the host can influence probiotic effects through both direct and indirect mechanisms. These differences in probiotic effects due to diet and genotype demonstrate that it is essential to investigate probiotics in a complex model to fully understand how they modulate host physiology in order to properly apply them to improve human health.

Regardless of how sketchy the current causal picture is of microbiota and mental health, probiotics are a commercial goldmine. They are the basis of an industry that already (in 2015) earns 35 billion dollars per year (Jabr 2017). To gain a closer view of the appeal of probiotics, we examined patenting trends for microbiota and probiotics. A very high proportion of microbiota/microbiome patents are for probiotics (see sect. 7 in the Supplementary Material). Commercial investment in probiotics is increasing (Jabr 2017; Olle 2013), as is academic patenting activity related to probiotic and other microbiota-based therapies (Supplementary Fig. S1). Nestlé, the biggest food company in the world, leads the way with probiotic patents and patent applications in the European Patent Office; Danone, another large food company with many dairy-based products, ranks fourth (Supplementary Fig. S2).

With its simple cause-effect hints ("take probiotics and cure yourself"), MGB research is likely to attract even more commercial attention and funding. Perhaps maintaining this appeal is part of the reason so many MGB studies repeat the basic recipe of probiotic-based intervention as the single "microbiome" method. In this research environment, single-study findings of no effect from probiotics are simply less likely to be published (although meta-analyses and systematic reviews with negative findings do find publishing forums), and the complex models urged by some researchers will have limited appeal. However, as the occasional commentator has noted (e.g., Olle 2013), focusing on a few classic probiotic strains – identified more than a century ago by much cruder methods – seems an unduly narrow focus given how microbiome research is normally about highlighting community-wide microbial diversity and interaction. But perhaps for this very reason probiotics remain popular. They enable straightforward experimentation, by appearing to cut through complex interactions and thus suggest that simple, non-harmful treatments are possible, even for conditions as resistant to conventional interventions as autism (de Theije et al. 2014). This simplicity is important for the public uptake of MGB and other microbiome research.

7.5. Science communication issues

Human microbiome research has captured the public imagination. It is a very popular professional media topic. A simple search for "gut microbiota" in the Factiva press database retrieves almost 1,500 publications. Even when narrowed down to a "microbiota gut brain" focus, the search still yields more than 300 press publications (see sect. 8 in the Supplementary Material, especially Fig. S3, for details). Less than a third of these press articles contain elements of caution or scepticism, and most are accompanied by very enthusiastic and optimistic claims. Generally, these articles make simple and encouraging reports on microbiome research and its potential impact on physical and mental health (e.g., "Pathogens in the stomach alter the brain's development and may increase an individual's risk of suffering from [autism] spectrum disorder" [Thompson 2017]). A common template is to highlight dietary change (including probiotics) as a "natural" means of changing the microbiome, and thus host health status (e.g., "Taking probiotics and adopting a gluten-free lifestyle may improve [autism] sufferers' social behaviour and ability to express emotions" [Thompson 2017]).

A valuable lesson for press releases about research can be learned from early microbiome research on obesity (e.g., Turnbaugh et al. 2006). Numerous studies, both experimental and bioinformatic, found associations between certain proportions of microorganismal groups and obesity. However, as these studies accumulated, this allowed meta-analyses and systematic reviews to be conducted, and these earlier findings fell away (Duvallet et al. 2017; Sze & Schloss 2016). Initial findings, although widespread, were from small samples, with hidden variations in background conditions (Schloss 2018). As we already noted, high inter-individual variability means large samples are required to make meaningful findings. Apparent effects in the obesity case turned out not to be real. Such developments in a new field are not surprising. It takes an accumulation of studies to allow meta-analyses to be conducted, and once they are, the field can correct itself.

However, even if a field manages to correct itself, systematic analyses of press articles have shown that public media material, including that produced by academic public relations offices, often focuses on initial spectacular findings. These early findings are often obtained from relatively small samples and are promissory rather than enduring (Gonon et al. 2011; 2012). Although early dramatic findings and press coverage can help attract funds to fledgling fields, and rapidly inform the public about potential avenues of treatment, the downsides are misinformation, unrealistic expectations, and eventual public and political backlash. The last is especially likely if initial findings cannot be translated into accessible therapies quite as readily as press releases might suggest (Hanage 2014).

But professional media are probably of less magnitude in this potentially misleading communication than is the large number of social media posts discussing microbiomes and health generally, and mental health in particular. Although we did not systematically survey blogs, tweets, and other such media, we did examine the first 50 Google hits for searches using gut + brain + microbiome (see Supplementary Table 8). Additionally, we performed a survey of Twitter posts of news articles in 2017 (see Supplementary Table 9). Although many of these online materials refer to actual research, they rarely do so critically. At most, they acknowledge that much more research has to be done. Notable exceptions within our small sample are an opinion piece cautioning against blanket belief in the efficacy of probiotics (DiSalvo 2017) and a book review raising questions about the simplicity of the "psychobiotic"

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approach (Fleming 2017; many reader comments are sceptical too).

The majority of the posts and shared news articles we surveyed suggest that new microbiome-related mental health treatments are just around the corner. Some websites and Twitter accounts promote probiotic and other dietary interventions as replacements for conventional psychiatric treatments. Many of these alternative "treatments" accord with standard nutritional and lifestyle guidelines (eat more fresh and less processed food, less fat and sugar, and more fibre; get more exercise; avoid stress). These are reasonable and no doubt helpful recommendations, regardless of how idiosyncratically some of them may be phrased on Twitter. What is concerning, however, is how this very ordinary dietary advice can be proposed as the solution to many mental health conditions. Even though clear cause-effect links between diet-altered microbiota composition and bodily or mental status are unknown, these gaps themselves leave room for the sentiment that it is all just "common sense" and that science is finally catching up to what everyone already knew in his or her gut anyway. Some MGB papers in the broader literature appear to endorse this way of thinking (e.g., Cowan et al. 2018) and may even sign up for other dubious health claims floating about in the public sphere. For example, using "leaky gut" language when it is not medically recognized as the basis of any disorder, let alone as a major causative agent of autism syndromes (Quigley 2016; Rao & Gershon 2016), is harnessing science to the fortune of what may be a medical fad.

As Perez-Muñoz et al. (2017) argue, when they debunk claims of in utero or placental colonization:

> Today, scientific findings can move freely from professional journals into the public realm (e.g., through social media), often before the scientific community has thoroughly discussed and vetted the evidence … it is our responsibility [as scientists] to debate these controversial topics and facilitate the self-correction process. Failure to do so may ultimately compromise human health, damage scientific creditability [*sic*], and potentially contribute to the erosion of the public's trust in science. (p. 15)

We suggest that human microbiome research in general (Hanage 2014) and MGB research specifically are at a point where careful reflection on the broader reception of the science would be highly appropriate.

8. Summarizing our findings

To its credit, MGB research is driven by hypothesis testing, but it mostly proposes and confirms loose conjectures about microbial involvement in brain and behavioural states. Microbiome research (outside MGB) is very technology driven and often fishes around after analysis for some sort of hypothesis that might reasonably be based on the data. Neither extreme of this continuum of practice is desirable for the maturation of microbiome research. In fact, we could see in MGB research the potential to integrate and balance these two ways of doing science. Very importantly, this merger would bring more microbiome depth to MGB research, which our analysis shows is missing and misunderstood.

We also showed how MGB research has many other compounding methodological and interpretive issues. But might all the issues we have identified just be signs of a young field? Will it not get better all of its own accord, given enough time? We agree it is important not to inhibit new approaches as they develop. But a strong foundation seems important for future development, rather than ongoing reproduction of a rough-and-ready approach. We have taken a critical approach to this emerging field, partly because we see the same claims repeated over and over again. They achieve a wider reach with every iteration. Using evolutionary, ecological, microbiological, neurological, immunological, biochemical, genetic, molecular, and developmental perspectives to bolster a narrow band of results both overreaches and also displays limited acquaintance with some of the well-established knowledge in these fields. These limitations matter not only for the future of a field, but also for the status of scientific activity in these challenging times. As we suggest and others have argued (e.g., Hanage 2014; Perez-Muñoz et al. 2017), overblown claims damage the credibility of the field and cause harm to the general social reception of science.

A topic worthy of further social scientific investigation is why microbiome research in general is so popular with the public, and whether public perspectives on microbiome research are changing how people think about health, including mental health. We speculate that reasons for the public uptake of microbiome research findings, including MGB, are to do with its perceived "naturalness" and the "holism" of the science, as well as the strong potential for microbiome-related therapies to be self-administered and even "DIY" rather than imposed by technical experts. There are many good aspects to any such trends. But MGB research should be aware of these tendencies and their possible relationships with anti-scientific claims (e.g., anti-vaccination; anti-psychotropic medication). It could be well worth working with relevant public health and media experts on how to communicate this exciting body of work responsibly.

9. Conclusions and future directions

Despite the critical picture we have painted, we see MGB research as a field full of promise, with important implications for understanding the relationship between the brain and the rest of the body. Existing MGB findings point to an ongoing need for more connected research that is able to investigate the complex interactions occurring in multipathway systems. Expecting cure-alls to emerge from these early days in which the puzzle pieces have barely been recognized, let alone joined up, seems contradictory to the spirit we assume to be motivating MGB inquiry. Our findings indicate the tension between a field-wide recognition of complex networks of causes and effects versus expectations of a simple all-efficacious treatment. As we noted in the introduction, our critical overview of MGB research is from outside the field itself and does not presume it can provide the detailed advice necessary to lead the field forward. This has to come from within the field. Nevertheless, we can use our findings of the current state of the field to propose some general pointers about how the field might develop and what it should avoid in that development.

9.1. What is known?

Perhaps the clearest general finding from MGB and the encompassing field of microbiome research is that microbiota are implicated in a wide range of ecosystem activities, some of which take place in human and other animal bodies and may be of considerable importance for understanding health and disease. Some of these connections are surprising, even if foreshadowed by earlier research (see sect. 2), and, if worked

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out experimentally and in clinical trials, could transform treatment options for ill humans. There do indeed seem to be links between microbes and mental health states, but they are extensively mediated by developmental, immunological, and metabolic processes that are in turn affected by environmental factors. Quite what these microbiome connections entail is the central question, and revealing the nature of any causal processes involving microbiota is what all MGB and other microbiome studies ultimately aim to do. Many researchers in MGB are now trying to fill the causal gaps and narrow down how microbiota or probiotics change mental health.

9.2. What is improving?

Several MGB and other microbiome papers in recent years have urged more rigorous experimental design, with appropriate positive and negative controls and adequate statistical power to allow the identification of cause and effect relationships and point to mechanistic explanations (e.g., Bruce-Keller et al. 2018; Lyte 2011; Schloss 2018). More sophisticated microbiota sampling and analysis will help us understand which groups of organisms are contributing to putative effects (Knight et al. 2018). Models that capture such interactions and their dynamics over time are going to be crucial, and some are already developed for broader microbiome research (e.g., Bucci & Xavier 2014).

Integrating multiple levels of causal influence in producing any kind of disease is always challenging, but if there is one thing microbiome research brings to the fore, it is awareness of the challenges in making causal claims about complex systems. The earlier rush to identify promising causal relationships in MGB research, and simplistically attribute large-scale effects to "the microbiome," or one-off probiotic interventions, can most constructively be understood as heuristic strategies that await more rigorous inquiry. There is now sufficient background knowledge to allow the refinement of hypotheses about microbiota relationships, and placeholder claims about causality can be put to the test.

9.3. What should be stopped?

Although we see many positive developments along methodological lines in MGB research, it is still accompanied by large helpings of overinterpretation, even if these come with a sprinkling of caution. Sometimes, it seems as if cautionary statements are used as liability limitation clauses in the ongoing promotion of the research (this is what we labelled in sect. 7 as "double-dipping"). Helpful as reviews may be to introduce non-experts to an emerging field, the wholesale marketing of MGB research in such a prolific review literature may "oversell" currently limited findings. Being more strategic about how the field is promoted, within and without science, could have long-run dividends that MGB researchers may want to consider.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0140525X18002133.

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Open Peer Commentary

Increasing reproducibility and interpretability of microbiota-gut-brain studies on human neurocognition and intermediary microbial metabolites

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Abstract

In this commentary, we point to guidelines for performing human neuroimaging studies and their reporting in microbiota-gut-brain (MGB) articles. Moreover, we provide a view on interpretational issues in MGB studies, with a specific focus on gut microbiota–derived metabolites. Thus, extending the target article, we provide recommendations to the field to increase reproducibility and relevance of this type of MGB study.

In a relatively new field, researchers have now started to use human neuroimaging techniques, like functional magnetic resonance imaging (fMRI), to study the microbiota-gut-brain (MGB) axis. A quick search with the terms "microbiome" and "fMRI" yields at least eight studies since 2013 that have linked task-related or resting fMRI to gut microbiota measures or interventions (Aarts et al. 2017; Ahluwalia et al. 2014; Bagga et al. 2018a; 2018b; Osadchiy et al. 2018; Pinto-Sanchez et al. 2017; Tillisch et al. 2013; 2017). However, out of those eight studies, only three based their analyses on groups of more than 20 participants, only two shared their neuroimaging data (in line with journal requirements), and only two (using an intervention) preregistered their design and analyses. As the field is still evolving, we would like to take this opportunity to make a plea for reproducible and interpretable MGB findings, pointing to guidelines for preregistration, results reporting, and data sharing in human neuroimaging studies and making suggestions to increase functional MGB interpretations, thus going beyond the many valid criticisms reported by Hooks et al. and actually providing recommendations.

Many MGB intervention studies register their human trials in a clinical trial register, but this is not common yet for observational MGB studies. However, it is important for reproducibility to preregister the main experimental question, hypotheses, design of the study, justification of the sample size, and the primary and secondary analyses. This limits the researcher's degrees of freedom and, hence, (uncorrected) multiple testing and presentation

of only desirable results (p-hacking) or post hoc hypothesis generation that is presented as a priori (HARKing) (Forstmeier et al. 2017). Preregistration also allows the presentation of null results, which are crucial for a field to develop. Naturally, journals play an important role in allowing null results to be presented and preventing publication bias. Registered reports (i.e., peer-reviewed preregistrations) are helpful for eventually reporting possible null results, as reviewers have deemed the design and sample size valid for answering the research questions (Chambers et al. 2014). Of course, findings based on unplanned and unregistered analyses are still relevant to generate new hypotheses, but these should be reported as exploratory and confirmed in future studies designed to answer this specific new research question.

Similar to gut microbiome data, fMRI data are characterized by high dimensionality, with thousands of voxels and, hence, thousands of statistical tests performed. Therefore, when reporting fMRI data, the type of multiple comparison correction should be clearly described (Poldrack et al. 2008). For cluster-based inference, the right use of the cluster-defining threshold is essential (Eklund et al. 2016).

Open science practices – such as sharing of data, analysis scripts, and preprints of publications – have many benefits, including easier replication, increased availability of data for theory building and meta-analyses, and increased possibility of review, before and after publication of an article. For human MGB studies using MRI, we can recommend the advice by the Committee on Best Practice in Data Analysis and Sharing (COBIDAS) (Nichols et al. 2017).

Increasing research on the gut microbiota, including MGB research, has also resulted in massive amounts of data being generated and shared (Editorial 2017). For example, consortia efforts such as the Earth Microbiome Project, Human Microbiome Project, Tara Oceans, and MetaHIT, as well as laboratory-level projects, have generated extensive data pools. Particularly, because of the size, complexity, and diverse formats that come with generating massive data, accessibility and accuracy of these data remain problematic to understand existing data sets. Increasing awareness of the different databases available for the data types and appropriate analyses to use (as listed online: https://www.nature.com/sdata/policies/repositories) could help overcome this obstacle. Equally important is to report how samples were collected, handled, and stored and what methodology was applied to analyze them, as these factors have a dramatic influence on the results.

Many MGB studies propose microbial metabolites as intermediary mechanisms that consolidate the link made between brain, cognition or mood, and microbes (Aarts et al. 2017; Bagga et al. 2018b; Osadchiy et al. 2018; Waclawikova & El Aidy 2018). However, bioavailability of microbial metabolites remains poorly understood. For example, there is scarce evidence on whether, when, how, and where these metabolites cross the epithelial barrier and blood-brain barrier and how tightly this process in regulated. Despite the remarkable progress in developing high-throughput techniques to identify microbial-derived metabolites, the majority are yet unidentified, and most of the identified ones remain functionally uncharacterized. The latter is related to the challenges of culturing bacterial species (Lagier et al. 2018). Although many bacteria in the gut remain uncultured, the current advances in the culturomic approach have enabled the culture of hundreds of new commensal bacteria, thus providing exciting new perspectives on their metabolic activity.

Another challenge confronting MGB studies is the interaction between gut microbes and dietary components – including precursors of neurotransmitters – and the effect on their metabolic products. Currently, all functional MGB studies have been limited to single or limited bacterial species and have not taken diet composition into account because of the extreme complexity of the gut organ system and technical limitations. Microbial-derived compounds, which are mainly products of their breakdown of diet, signal not only to the host cells, but also to other gut bacteria in a beneficial or adverse way (Adair & Douglas 2017). Considering that more than 1,000 different bacterial species are estimated to reside in the human gastrointestinal tract, this gives an enormous amount of possible variations in inter-microbial communication by produced metabolites (Postler & Ghosh 2017). One way to facilitate interpretation of the complex MGB interactions and ultimately allow new therapeutic approaches to treat MGB-related disorders is by developing high-throughput gut and brain organoid systems obtained from urine and blood samples of individuals and cultured with stool samples of the same individual (Dutta et al. 2017). Results could be used to explain inter-individual differences in human neurocognition and its response to – for example, nutritional or pharmacological – interventions, providing a functional and interpretable MGB link.

The parent-offspring microbiome and neurobehavioral development

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Abstract

We identify the significance and typical requirements of developmental analyses of the microbiome-gut-brain (MGB) in parents, offspring, and parent-offspring relations, which have particular importance for neurobehavioral outcomes in mammalian species, including humans. We call for a focus on behavioral measures of social-emotional function. Methodological approaches to interpreting relations between the microbiota and behavior are discussed.

No single analysis can adequately cover all facets of a complex interdisciplinary field, but we believe there remain a few topics that warrant inclusion in the present discussion of microbiota-gut-brain (MGB) research. Foremost is *developmental analysis* of the MGB in parents, offspring, and parent-offspring relations. Development is arguably a necessary element in understanding

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any biological system. None of the "25 most cited" papers (Table 1 in Hooks et al.) comprise developmental analysis, that is, studies designed to elucidate pathways or processes by which the offspring gut is inoculated with bacteria and in which we see the MGB and behavior emerge and differentiate in interaction with their surroundings. Such analyses would include whether and how the stages of microbiota development stimulate neural, neuroendocrine, and behavioral activity at corresponding stages and that, in turn, reciprocally affect the gut bacteria. Another, important aspect of mammalian development is parent-offspring interaction: whether and how a parent-offspring microbiome may form and especially how the microbiota of each individual affects the other, with routes including behavior that is influenced by individual and familial microbiomes.

We emphasize that performing a manipulation at an early age and measuring effects at a later age or ages does not provide "developmental" information. Likewise, manipulations at various time points can produce different results at different life stages, but again, this says little about process or pathways. Such factors and myriad cascading and interactive events could contribute mechanistically to the development of disordered social behavior.

Another non-deferrable topic is the need for more integrative and informed use of *behavior as a measure and as a mechanism*. Hooks et al. appropriately identify some of the limitations of the handful of behavioral measures currently used to model social-emotional health and disease. Now is a time to discuss how to validate and apply behavioral measures as more precise and informative metrics of function, especially in social-emotional processes. We need to acknowledge the import and the sophistication of careful behavioral analyses. Needed are methods that are sensitive, objective, and sufficiently nuanced to capture social behavior and the nebulous emotional forces that shape and help regulate it. This need is not unique to MGB investigations; it is widespread in the life sciences, where we seek to understand relations among genetic, cellular, system-level, and organismal functions. The MGB as a system is observed and evaluated via behavior, so improved behavioral tools will deepen our ability to see and measure microbiome effects. In developmental contexts, relevant behavioral methods include those designed specifically to titrate dyadic interactions, particularly for mammalian species where internal gestation and extended parental care set the stage for continuous, dynamic interactions. Parent-offspring interactions include behaviors that can modulate and shape social behavior. We must not only measure behavior, but also recognize it as an active agent in development.

Parental behaviors are associated with offspring neural and endocrine development and with epigenetic impacts, and they are themselves affected by the offspring. Sibling interactions are similarly influential. Include microbiota effects and the picture is even more functionally integrated. Such interdependencies prevent individual offspring within a litter from serving as statistically independent observations (e.g., Abbey & Howard 1973).

Hooks et al. define the goal of MGB research as understanding the characteristics of the microbial community in bidirectional regulation with the gut-brain axis and the mechanisms by which such influence occurs. They question whether MGB research is validly advanced by some common approaches, including the use of germ-free (GF) animals. Their characterization of the purpose and use of GF animal models is limited. Sophisticated, experimental use of GF animals is critical and includes testing the impact of specific colonizing microbiota, for example, the microbiota of healthy versus affected individuals on an animal's phenotype, as well as interventions that rescue the phenotype. Controlled colonization of GF animals can enhance study of the tissue-specific role of characterized microbiota – a line of study that Hooks et al. rightly indicate should be better developed in MGB research. Further, they ask: Is the goal of MGB research to identify the impact on neurobehavior of a specific causal organism or a microbial community? In our view, MGB research rightly includes both approaches. That is, while considering the microbial community in its entirety, it is valid and important to determine whether specific members of the microbial community are missing or over-represented in affected individuals compared to healthy controls, and whether this difference can alone explain the phenotype of interest. This direction of inquiry helps refine understanding of mechanism and targets potential therapeutic approaches.

We wholly concur that advances in MGB require application of state-of-the-art measurement using well-validated methods of DNA profiling of microbial communities. Current methods are nicely described by Knight et al. (2018) and involve either amplicon sequencing using a specific region of the bacterial 16S rDNA gene or shotgun sequencing. The former provides rapid, low-cost characterization of bacterial abundance and diversity. Shotgun sequencing deepens the data obtained from each sample by characterizing all genes, whether bacterial, archaeal, viral, or eukaryotic. Further advances are occurring through use of complementary methods that increase insight into what the microbiota are doing (i.e., their functions). For this reason, pairing metagenomic analysis with other -omics, such as metabolomics, metatranscriptomics, or proteomics, is highly recommended and should be incorporated into MGB research whenever possible.

If we are to address disorders of human behavior, we must devise tests that identify a dysregulated and a regulated healthy mouse, so we can better see the shared mechanisms that make a dysregulated and a regulated, healthy person. Despite their valuable observations and critiques, we were disappointed that the authors called for more justification for using data from nonhuman animals to penetrate some of the mysteries of human disorders. They seem to lack appreciation for the power gained from recognizing evolutionary conservation of core mechanisms. Indeed, we think that expanding the use of diverse species into MGB research will contribute importantly to our understanding of behavioral diversity in the natural world (Ezenwa et al. 2012), as well as enhance our translational insight into MGB phenomena in humans.

Microbiota-gut-brain research: A plea for an interdisciplinary approach and standardization

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Abstract

Hooks et al. note that microbiota-gut-brain research suffers from serious methodological flaws and interpretative issues. We

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suggest two corrective measures: first, taking more seriously the need of interdisciplinary work; second, interpreting some of the methodological issues as ordinary challenges of standardization, typical of emerging disciplines.

In their article, Hooks et al. propose a critical analysis of microbiota-gut-brain (MGB) research showing that it has many methodological flaws and interpretative issues. Although we think that the authors' arguments are generally well founded and embraceable, we argue that some of the major issues are typical of an *emerging multidisciplinary* field of research. As is the case with many other disciplines, microbiome research has committed some sins of youth, mostly attributable to the eagerness of publishing new concepts in a field that is dominated by financial interests. On the one hand, this has attracted new funding. On the other hand, it has generated many expectations both from an industrial point of view and from the social community. For these reasons, in the attempt to be pragmatic, we suggest two corrective measures.

First, we suggest that neurobiologists, immunologists, or microbiologists *seek advice* from scientists in the multidisciplinary fields related to the research, to limit misinterpretation or generalizations. Today, biomedical sciences are increasingly complex, often requiring an intertwined methodological repertoire and knowledge in several fields. The same revolution has hit other areas of microbiota research. Mucosal immunology, for example, has witnessed an injection of scientists, including microbiologists or non-mucosal immunologists, that had no clue as to what an immunoglobulin A or epithelial barrier was. Still, after careful reconsideration of some misleading concepts or interpretations, the benefit of such an injection has arrived, including the awareness of how the microbiota may shape immune responses also at distant sites. In the last few years, next-generation sequencing and the development of meta-omics approaches (e.g., metagenomics, metatranscriptomics) have allowed us to investigate the composition and the modulation of gut microbiota. This is important as only around 1%–2% of bacteria can be cultured in the laboratory, which has led to the proliferation of correlation studies without any functional relevance of the "putative" correlative strains. Culturomics (i.e., the art of culturing microorganisms) has thus been rejuvenated, and many groups are now trying to isolate strains of interest and to demonstrate a "cause-effect" relationship.

Second, we suggest interpreting some of the major issues identified by the authors as ordinary challenges of *standardization*. As many sociologists of science have shown, standards are a necessary condition for the very existence of contemporary science, and "standards-setting activities" are legitimate scientific tasks (e.g., Goodrich et al. 2014). Hence, we should not overestimate problems that are part of an ongoing process, typical of emerging disciplines. To illustrate this point, let us restate some of the methodological issues of MGB research in terms of standards (see Timmermans & Epstein 2010).

Design standards define the properties and features of experimental systems. Researchers need robust mouse models to perform studies investigating causal roles of the gut microbiota on the host. Several of them are available, such as germ-free (GF), humanized gnotobiotic, and specific pathogen-free (SPF) mice. All of them allow us to evaluate the impact of microbiota on an experimental question. However, each comes with its own drawback. For example, GF mice have an immature immune system and behavioral defects, thus affecting the outcome when the scientific question relies on the involvement of the immune or neurologic system. Gnotobiotic and SPF mice are susceptible to the high variability of microbiota composition attributable to environmental factors (Hugenholtz & de Vos 2018). Therefore, it is important that several models are used simultaneously to minimize bias. This strategy would allow us to address more precisely the role of microbiota in a given experimental question (Rescigno 2017), but it would require a greater financial and cognitive effort.

Terminological standards ensure the stability of meanings across researchers and over time. But scientific terms are constantly evolving, and their use is often a matter of establishing a convention within research communities. *Gene*, for example, has been extensively revised over the years, and still there is not a unanimous definition (Boem et al. 2016). As the authors know (see Hooks & O'Malley 2017), the idea of identifying a wild-type microbiota, one in which gut bacteria exist in an evolutionarily optimized form, has been abandoned already in favor of a functional account (Lloyd-Price et al. 2016). Nonetheless, recent work on relevant probiotic strains has also led to the isolation and characterization of certain soluble factors secreted by live bacteria called *postbiotics*. These studies suggest that postbiotics may contribute to the host health by improving specific physiological functions (see Tsilingiri & Rescigno 2012). Likely, an improved understanding of the exact mechanisms of postbiotic activities might contribute to grasping the impact of dysbiosis on disease development, bypassing the complexity of individual variability in microbiota ecology (Levy et al. 2017).

Performance standards set outcome specifications. For example, statistical significance specifies the level of acceptability of an experimental result. However, the use of statistics in biomedical sciences has been harshly contested in the last few years. Misuse and misinterpretations of null-hypothesis testing have led to many non-reproducible and inconsistent findings in many disciplines, including biology (e.g., Nosek & Errington 2017). The challenges seem to be widespread and pervasive enough to warrant immediate and careful attention. Lowering the threshold of statistical significance is just a proposal (see Benjamin et al. 2018), which also has been largely contested (e.g., Ioannidis 2018). As of today, the standard of statistical significance for claiming a scientific finding remains fixed at 0.05, and it is unlikely to change soon. Nonetheless, the development of more accurate performance standards is a need for the entire field of biomedical research.

In conclusion, there is no denying that microbiota research, although still in an "early phase," can contribute to improving our understanding of biology in all its aspects. We should expect its findings to improve as the standards are settled and collaboration between scientific departments is improved.

Beyond a gut feeling: How the immune system impacts the effect of gut microbiota in neurodevelopment

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Abstract

Hooks et al. posit that gastrointestinal microbes alter the end state of development indirectly. Here, we present the immune system as the link that facilitates communication between the gut and the brain. Illustrating the case of autism spectrum disorder, we explicate the role of the immune system in responding to microbial dysbiosis by inducing an inflammatory state that affects neurodevelopment. We propose two models: directly, within the infant, and indirectly, via maternal and infant systems.

The gut microbiota has been recurrently reported to influence developmental outcomes (e.g., Finegold et al. 2002; Kang et al. 2013). Hooks et al. proposed that the mere presence of microbes in the gut triggers a response in the developing organism that results in an altered end state of development, rather than the microbes being a direct causative agent. Although we concur with this point, it is important to further address it in relation to the immune system and how pivotal it is as an intermediary system that affects neurodevelopment (Fig. 1). To illustrate our point, we present the case of autism spectrum disorder (ASD), a neurodevelopmental condition that features restricted behaviors and deficits in social communication (American Psychiatric Association 2013).

The comorbidity of ASD and gastrointestinal disorders has been often reported (e.g., Ashwood & Wakefield 2006; Torrente et al. 2002). In our recent review, we showed that the risk of ASD increases with respect to the inflammatory state, rather than to the presence of a specific species of microbe, underscoring how system-wide changes in inflammatory profiles may direct neurodevelopmental trajectories (Azhari et al. 2018). Indeed, the response to gut microbiota that the organism launches is primarily composed of signals from immuno-inflammation pathways, allowing for a relay between the gut and the central nervous system (CNS) (Carabotti et al. 2015; Erny et al. 2015). This gut-immune-brain communication can be traced to several main points of contact across the systems, primarily facilitated by signaling immune molecules. The gastrointestinal microbiota has significant influence over the profile of certain circulating pro-inflammatory cytokines, chemokines, and growth factors, such as IFN-γ (interferon-γ), IL-17 (interleukin-17), IL-6 (interleukin-6), and TNF-α (tumor necrosis factor-α) (e.g., El-Ansary & Al-Ayadhi 2014). The link between autism and immune dysfunction has been asserted by findings of atypical upregulation of these cytokines in persons with autism (e.g., Khakzad et al. 2012; Li et al. 2009).

Pro-inflammatory cytokines function as signaling molecules and have the capacity to communicate with the CNS, serving as a bridge between the gut microbiota and the brain. Indeed, post-mortem studies on brain tissues obtained from deceased persons with autism showed the presence of enhanced neuroinflammation in several brain regions, including the cerebral cortex, white matter, and cerebellum (e.g., Vargas et al. 2004). Pro-inflammatory

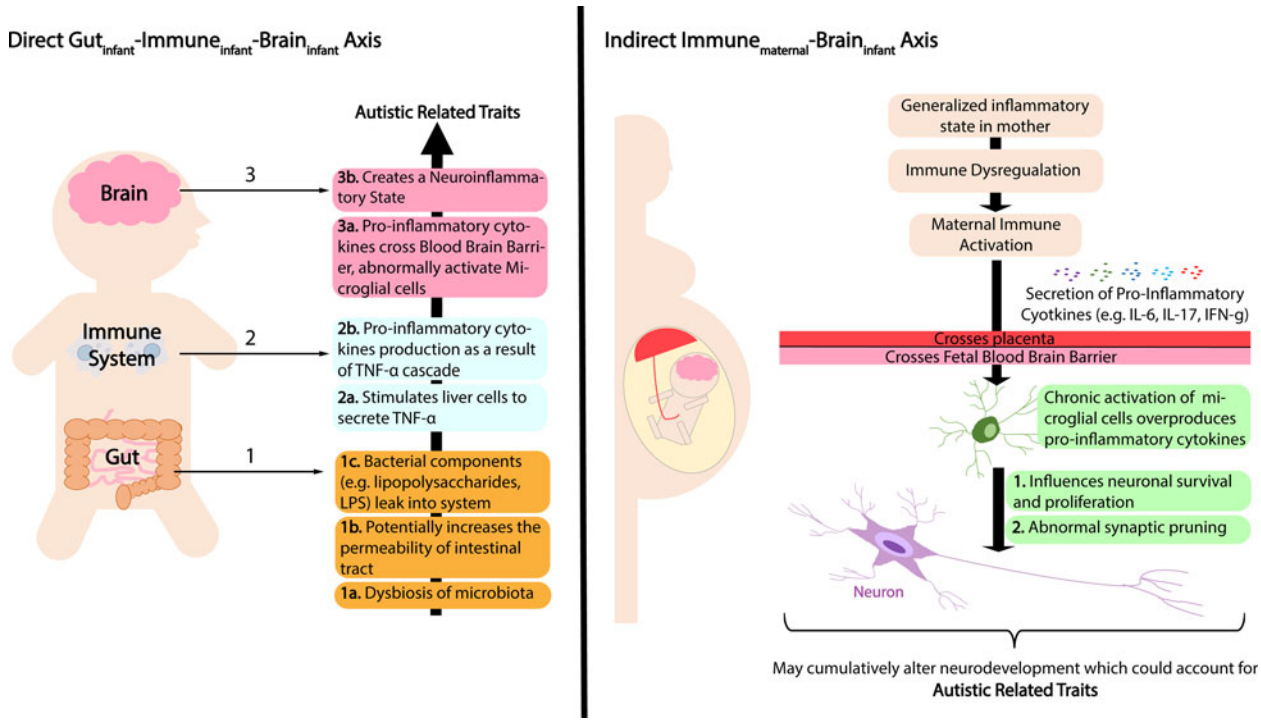


Figure 1. (Azhari et al.) Schematic diagram illustrating theoretical models of direct (gut$_{infant}$-immune$_{infant}$-brain$_{infant}$) and indirect (immune$_{maternal}$-brain$_{infant}$) mechanisms underlying autism spectrum disorder (ASD). The direct model features atypicality in the infant's gastrointestinal microbiota, leading to system-wide neuroinflammation in the infant. The indirect model features dysregulated maternal gastrointestinal microbiota, instigating maternal immune activation (MIA) and subsequent neuroinflammation in the infant. Neuroinflammation stemming from either of the two mechanisms will compromise neurodevelopment and induce the emergence of autistic-like traits.

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molecules responsible for this have been postulated to impede brain development indirectly, through non-neural cells. In individuals with ASD, a type of resident non-neural cell that has been found to be activated at atypical levels constitutes the microglia (e.g., Tetreault et al. 2012). Although moderate activation of the microglia in response to injury or infection in the CNS is protective, chronic microglial activation compromises brain development in one of two ways. First, abnormally activated microglia overproduce pro-inflammatory cytokines, which contributes to damage in synaptic networks and neuronal cell death (Rodriguez & Kern 2011). Second, chronically activated microglia are responsible for elevated occurrences of phagocytosis and excessive removal of neuronal debris, leading to impaired neural development (Takano 2015). Dysfunctional microglia are no longer sensitive to external immune signals either, which leads to a perpetuation of dysregulated phagocytosis (Fernández de Cossío et al. 2017). At present, accumulating evidence drives at one possible hypothesis: In autistic persons, elevated levels of pro-inflammatory cytokines may stem from the instigation of the gut microbiota onto the immune system; these immune signals contribute to neuroinflammation that ultimately hinders neurodevelopment. This theory posits a gut_{infant}-$immune_{infant}$-$brain_{infant}$ model, where biological pathways from the gut microbiota to the eventual emergence of the autistic phenotype occur within an individual. Although this hypothesis is intriguing, at present, researchers have yet to elucidate a causal pathway that proves that the dysregulation of pro-inflammatory cytokines attributable to dysbiosis of the gut microbiota is the same immune phenomenon that leads to chronically activated microglial cells in the brain of ASD individuals.

As neurodevelopment begins early in the course of fetal maturation, it is important that we address the mechanism at the prenatal phase too. Hooks et al. refuted the postulation of the gut microbiota exerting any neurodevelopmental impact given that the in utero environment is sterile. Although the notion of in utero sterility may be true, the immune system of the mother, however, could still impact the fetus during gestation. We posit that the state of generalized inflammation in the pregnant mother, maternal immune activation (MIA), potentially alters neurodevelopment in infants (e.g., Gilmore et al. 2005). This theory has largely been supported by animal studies, such as that conducted by Kim et al. (2017b). In this mice study, the authors showed that specific maternal gut microbes are associated with an increase in pro-inflammatory IL-17 in the mother, along with the appearance of autistic behaviors in the offspring. A study on ASD patients showed that TNF-α, potentially secreted by liver cells in the presence of gut lipopolysaccharides (LPS), creates a peripheral inflammation that results in microglia activation in the brain (e.g., Breese et al. 1994; Qin et al. 2007). As opposed to the first model, this second model presents an indirect $immune_{maternal}$-$brain_{infant}$ pathway that involves cross-talk between the immune system of the mother and the CNS of the infant.

In conclusion, we have presented the case of ASD as a neurodevelopmental condition involving both the gut microbiota and the immune system. We have also proposed two theoretical models that the field should consider (Azhari et al. 2018). The first model features direct gut-immune-brain association within the individual at the postnatal phase, whereas the second model is an indirect model implicating both maternal and infant systems in the prenatal phase. These theoretical models may sprout experimental paradigms that allow for existing postulations to be tested and, in doing so, uncover causal pathways from the gut to the immune system and, ultimately, to the brain.

Stress and microbiota: Between biology and psychology

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Abstract

This comment expands on Hooks et al.'s criticism of the problematic and overly general uses of "stress" within the microbiota-gut-brain field. The comment concludes that, for the microbiota-gut-brain field (as for other fields drawing on "stress"), much work is yet to be done in terms of how we explore and understand biology vis-à-vis psychology.

Hooks et al. note that approximately half of the studies surveyed explore relations between the "neuroendocrine stress axis" and microbiota. They criticize these studies for translating too easily between animal models and humans, and they argue that the measurement of stress via hormones such as corticosterone is potentially confounded by other factors. These criticisms are sound, but with stress as such a central concept to parts of microbiota-gut-brain research (and beyond), it is central to expand on them, because these criticisms illuminate the difficulties of exploring neurobiology vis-à-vis psychological states and behaviors such as "stress."

Across the papers surveyed by Hooks et al., stress is first deployed with a considerable methodological and conceptual diversity. The papers report both "stress" and stress that is, for example, "chronic psychological" (Ait-Belgnaoui et al. 2014), "acute" (Crumeyrolle-Arias et al. 2014), "acute psychological" (Ait-Belgnaoui et al. 2012), or the result of "exposure" to a "social stressor" (Bailey et al. 2011). Presumably, these qualifiers specify the type of stress explored, yet they rely on no coherent theoretical paradigm and employ very different methods of inducing "stress."

For example, one paper (Ait-Belgnaoui et al. 2012), uses the notion of "acute psychological stress" in their paper's title, a term that surprisingly shifts into "partial restraint stress" in the paper itself. This was induced by wrapping rats in constricting tape to limit (but not prevent) their movements for 2 hours (Ait-Belgnaoui et al. 2012, p. 1886). In another paper by the same team (Ait-Belgnaoui et al. 2014), "chronic psychological" stress is induced by placing rats on a small platform in the center of a tank filled with water, for 1 hour at a time, 4 days in a row. It is unclear, however, how qualifiers such as "psychological," "acute," or "chronic" actually connect to these methods. We may note, for example, that "acute stress" was induced for 2 hours, whereas "chronic stress" was induced for half that time (but repeated over 4 days). Other examples of "stress"-inducing methods include restraining mice in tubes for 1 hour (Sudo et al. 2004) or putting an aggressive mouse into another mouse's

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cage (Bailey et al. 2011), seen as a "social" stressor. One wonders why the confrontation between two mice is "social," whereas the handling of rodents by scientists does not classify as such (especially when research has shown that olfactory exposure to men increases the stress response in mice [Sorge et al. 2014]).

More generally, it is by no means clear if these types of stress are comparable to each other: Being physically constrained in a tube is different from being slightly restricted in movement (yet both are classified as "acute" forms of stress), which is different from being exposed to water for days in a row. Further, it is not entirely clear if whatever the rats go through is in any way comparable to stress in humans. As Hooks et al. note, most researchers attempt to avoid anthropomorphizing their rodents, hence using terms such as *anxiety-like*. However, no-one uses *stress-like*. It is simply assumed that the rats are experiencing stress comparable to that of human beings (see also Rose & Abi-Rached 2013, p. 97). Built into rodent stress research is thus a tacit assumption that there is a direct line from the stress of a rodent to the stress of a human being. Besides such translational problems between rodents and humans, there is also a circular reasoning employed, with the a priori assumption that the experimental paradigm (e.g., restraining mice) is stress inducing, and, accordingly, a following neuroendocrine response must thus reflect the induction of stress. This common line of thinking, however, contradicts another common assumption within stress research – namely, that stress depends on the organism's perception and appraisal of the experimental stimuli *as a stressor* (e.g., McEwen 1998; McEwen & Seeman 2006). Some researchers have suggested that the term *stress* should therefore be limited to conditions wherein organisms perceive a serious, unpredictable, and uncontrollable threat to their health (Koolhaas et al. 2011, p. 1292). Thus, tacking terms such as *psychological* or *acute* onto the "stress-inducing" experiments such as those noted previously only adds to a considerable conceptual and methodological confusion.

This also means that the measurement of hormones as indicative of "stress" is not just plagued by confounders, or circularity, but more fundamentally with the difficult problem of *what it means* when a rodent (or a human) experiences an increase in the activity of the hypothalamic-pituitary-adrenal (HPA) axis.

In humans, for example, one study shows how secretions of cortisol vary in experienced versus novice skydivers (the former's response is smaller and trails off more quickly), whereas their *experiences* of anxiety, before the jump, are similar (Meyer et al. 2015). If the association between subjective experience and hormonal response is equivocal, then the assumption that a hormonal response to a stress-test paradigm necessarily means that "psychological stress" has been induced is flawed.

There are, in sum, questions remaining over how we induce "stress" in animal models, of how we should analyze and interpret animal behavior, and, crucially, how this translates to the complexities of human experience and psychiatric disorders. These difficult questions go beyond microbiota-gut-brain research, relating more broadly to how we think about the "psychological" vis-à-vis the biological. Often, one will marvel at the complexity that is afforded to exploring, describing, and modeling biological pathways and mechanisms (in this case, microbiomes, guts, and brains) – yet it is peculiar that so persistently, much less effort is made to explore the psychological and behavioral beyond the most basic level of assumption and description. What is necessary are not declarations of how microbiomes "challenge our concept of self" (e.g., Rees et al. 2018) but much more careful and detailed explorations of microbiomes, guts, and brains *in relation to* careful operationalizations of behavior and the psychological.

Combining integrated systems-biology approaches with intervention-based experimental design provides a higher-resolution path forward for microbiome research

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Abstract

The Hooks et al. review of microbiota-gut-brain (MGB) literature provides a constructive criticism of the general approaches encompassing MGB research. This commentary extends their review by: (a) highlighting capabilities of advanced systems-biology "-omics" techniques for microbiome research and (b) recommending that combining these high-resolution techniques with intervention-based experimental design may be the path forward for future MGB research.

We generally concur with the descriptions by Hooks et al. on microbiota-gut-brain (MGB) research: There are numerous literature reviews, scientific conclusions are often subjected to over-interpretation, and there is a lack of detail about the microbiome itself. The goal of the present commentary is to recommend advancements in experimental design that could enhance microbiome research.

The four top-cited papers considered by Hooks et al. used microbiome intervention to demonstrate a neurological effect or association. Two papers described a germ-free model (Heijtz et al. 2011; Sudo et al. 2004), whereas the other studies used a probiotic intervention (Bravo et al. 2011; Hsiao et al. 2013). These studies effectively demonstrated that the microbiome can cause changes in neurological function and behavior of the host. Although two of these studies provide information about microbiome composition, via culturing or 16S rRNA sequencing (Hsiao et al. 2013; Sudo et al. 2004), this level of experimental design only provides general information about the composition and diversity of the microbiome, while failing to capture details about functional activities of this dynamic microcosm. The primary focus of these studies is to connect the microbiome to a specific physiological effect in the host. This is a critical first step in identifying whether a host

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symptom is affected by the microbiome but is insufficient to ascertain important details of the specific cause.

Deriving a more detailed understanding of how the metabolic activities of microbial communities affect host physiology requires an expanded experimental design. Many studies have demonstrated that microbial community composition varies across individuals but clusters as a function of age, geography, diet, co-habitation, and health (David et al. 2014; Halfvarson et al. 2017; The Human Microbiome Project Consortium 2012; Song et al. 2013; Yatsunenko et al. 2012). This implies that all these factors have an impact on the host's microbial composition, and that replicating the composition of the microbiome without exact replication of experimental conditions (e.g., individuals, laboratories, and diet) is very difficult. This is a formidable problem even with large sample sizes. This limitation suggests that there is less of a reproducibility crisis (Sze & Schloss 2016) and more of a need to design more elaborate and appropriate experiments (Knight et al. 2018).

There is now published evidence that despite significant variation in microbial composition, broad biological function appears to remain similar (The Human Microbiome Project Consortium 2012). Hence, microbiome research will be greatly propelled by experimental designs that combine intervention studies with systems-biology techniques that seek to characterize overall microbiome function. Currently, there are four "-omics" techniques that capture "detailed" molecular-level information about a microbial community: whole community genome (metagenome) sequencing (The Human Microbiome Project Consortium 2012), metatranscriptome sequencing (Jorth et al. 2014), metaproteome identification (Erickson et al. 2012), and metabolome characterization (Karu et al. 2018). Each of these techniques has advantages and limitations. However, combination of these techniques should lead to high-resolution portrayal of microbial function and, potentially, microbial metabolic reconstruction at the community level.

Whole metagenomic sequencing provides a backbone for investigating microbiome function via creation of a composite gene catalog. Initially, reference sequence databases were used to characterize the taxonomy and function of a microbial community (The Human Microbiome Project Consortium 2012; Stewart et al. 2018) but were limited by the range of known reference genomes and functional ortholog databases. Advancements in the understanding of genomic features allow combining tetranucleotide frequency with sequence coverage to bin the results from metagenomic assemblies into metagenome assembled genomes (MAGs) de novo (Kang et al. 2015). This approach provides a set of low- to high-quality draft genomes, which can provide subspecies and strain-resolved information about the gene repertoire of a microbial community (Brown et al. 2018). Although metagenomic sequencing allows categorizing a set of genes into their respective taxonomy and predicting their function, metagenomics is limited to only predicting potential function because it contributes no information about actual translation of genes into proteins.

Although the laboratory technique behind metatransciptomics is more similar to metagenomic sequencing, metatranscriptomics and metaproteomics explore a similar question: Which predicted genes are being transcribed and subsequently translated into proteins? Transcriptome sequencing identifies genes that are being transcribed into RNA and generally produces more total identifications, whereas liquid chromatography tandem mass spectrometry (LC-MS/MS)–based metaproteomics ultimately identifies proteins, thus providing confirmation that a metagenomics predicted gene is translated into protein (Erickson et al. 2012; Jorth et al. 2014). Although the number of identified proteins tends to be fewer than transcripts, it is unclear how many proteins microbes actually use in a community. Strides have been made by integrating MAGs with metaproteomes in lower-diversity communities, but much work remains to answer this question (Xiong et al. 2017). Theoretically, metaproteomics should provide a better connection to phenotype because functional products of gene expression are actually measured.

Gas (GC) and liquid (LC) chromatography paired with mass spectrometry provide an approach for detecting metabolites within microbial communities (Karu et al. 2018). Although these molecules are difficult to connect to their organism of origin without complementary gene information, metabolomics provides a framework to evaluate metabolic hypotheses derived from genetic or protein information. Enzyme-to-compound databases, such as Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al. 2016) and MetaCyc (Caspi et al. 2018), can annotate recognized genes with an enzyme identifier, thereby connecting specific taxa, genes, and proteins to specific molecular functions at the compound level. Ultimately, these features can create a framework by which metabolomic data can be integrated with microbiome data.

Using constrained experimental conditions combined with specific hypothesis-driven intervention, researchers can join the power of cause-and-effect experimental design with integrated "-omics" to fully characterize the microbiome under different interventions. Time-course studies would be ideal because the subject can function as its own internal control. Eventually, as the community of investigators transitions into the next phase of microbiome research, general intervention-based studies and broad characterization of microbial composition at the population level will no longer be sufficient to provide novel insights into the MGB axis. Holistic metabolic modeling through systems-biology measurements of individual microbiomes combined with intervention-based experimental design provides a path forward.

The contribution of microbiology to neuroscience: More complex than it seems?

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Abstract

The overblown, somewhat dramatic media interpretation of microbiota-gut-brain literature is highly misleading. This phenomenon is not new to neuroscience, wherein rapidly evolving research fields struggle to translate findings into clinical practice. Advances in microbiology might integrate our understanding of complex biological pathways that should be interpreted within neuropsychiatric symptom dimensions rather than specific disorders.

In the target article, the authors observe that microbiota-gut-brain (MGB) research has gained enormous public attention. We share

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their concern that public accounts of MGB research tend to force preliminary findings into over-the-counter probiotics or dietary suggestions to improve a broad variety of health outcomes. This appears to reflect a growing appreciation of natural lifestyle habits to oppose the perceived detrimental effects of increased urbanization and declining biodiversity worldwide. The MGB media hype should be considered in line with the sensationalized reporting of neuroscience that complicates social engagement and outreach (Illes et al. 2010). Oversimplification of highly complex research and misinterpretation of cardinal concepts such as correlation, causation, and association from widespread media resources can lead to overblown conclusions that negatively impact public health policy. Academic pressure towards public engagement for professionals without specific training in science communication is also likely to contribute. However, the authors' observations should be interpreted within the broader scope of current shortcomings in translating available biological data to the clinical field of psychiatry. The unprecedented acceleration of neuroscience technology over the past few decades has produced a critically large amount of knowledge that struggles to find its way into everyday practice. The difficulty of replicating findings across patient populations and the lack of significance at the individual level are thought to play a major role in slowing translation (Kapur et al. 2012).

People with severe mental illness (SMI) often show poor nourishment, and the relationship between dietary quality (and possible nutritional deficiencies) and mental health has recently begun to evolve beyond the classical field of cardiometabolic risk studies (Firth et al. 2018). One of the mechanisms under scrutiny is the gut-brain axis, a well-demonstrated key player in the combination of activity from immune, endocrine, metabolic, and neural pathways. The potential impact of commensal microorganisms on behavioral symptoms has been extensively studied in patients with irritable bowel syndrome, who often experience co-morbid anxiety and depression. Indeed, several different treatment options, focusing on microbiota manipulation, have been shown to be useful in contrasting the behavioral symptoms in animal models of the disorder (Clarke et al. 2012). Although some studies reported substantial effects of probiotics on emotional processing in healthy women (Tillisch et al. 2013), a recent meta-analysis concluded that findings from animal studies have yet to be confirmed in humans (Reis et al. 2018). In neurodevelopmental disorders, several papers recently addressed the question of whether reduced or altered microbial community could affect disease phenotype. Besides changes in microorganisms inhabiting the gut of patients, microbial metabolites have been suggested to actively participate in modulating the clinical severity, but their role is yet to defined (Borghi et al. 2017; De Angelis et al. 2013; Kang et al. 2018).

In women with anorexia nervosa, a significantly lower total amount of bacteria has been observed compared with age-matched healthy control subjects (Morita et al. 2015). Moreover, these patients showed an altered intestinal microbiota composition, with an unbalanced gram-positive/gram-negative ratio. Modifications in the abundance of microbial communities led to changes in the quantity/quality of microbial metabolites, decreasing the concentration of fecal butyrate, which was found to be negatively related to anxiety and depression symptom scores (Borgo et al. 2017).

The gut-brain cross talk has also been investigated in a limited number of patients with schizophrenia at clinical onset (Schwarz et al. 2018) or in the chronic phase (Shen et al. 2018). Both studies found significant changes in patients' microbial composition compared with control samples.

In our view, MGB research is still in its infancy and should be considered a highly promising tool to disentangle pathways that lead to increased risk for neuropsychiatric syndromes rather than causative in any way. In this light, novel and exciting work has begun to define a mediating role for gut microbiota in well-known mechanisms of neurodevelopmental impairment during pregnancy (Kim et al. 2017b). Not unlike other pathogenetic pathways that have been found to overlap across clinical syndromes, MGB research might gain significance within the dimensional Research Domain Criteria (RDoC) framework proposed by the National Institute of Mental Health to overcome the broad overlap of genetics, endophenotypes, and clinical symptoms across disorders (Cuthbert 2014). Findings on microbial gut abnormalities are likely to be explained within a network of individual–environment interactions and clinically defined symptom dimensions rather than specific diagnoses.

Finally, although newer technologies have allowed us to tremendously increase our knowledge on the commensal microbial community, Hooks et al. argued that "standard microbiome analyses are not carried out even in many of the most highly cited MGB papers" (sect. 5, para. 4). The authors' methodological approach, based on selecting the most highly cited papers, resulted in a list of studies ranging from 2004 to 2015. The chosen search methodology left out many papers that employed newer technologies such as 16S rRNA sequencing, shotgun metagenomics, and metabolomics. Furthermore, seven out of nine of the most recent included papers (2013–2015) were sequencing based. Even the most sophisticated technologies (i.e., shotgun metagenomics, allowing both taxa identification and functional characterization of the microbial community) often result in data that are difficult to interpret, highlighting the need for new tools of data integration and correlation (D'Argenio 2018). In this light, MGB studies appear to be limited by the intricate relationship between the central nervous system and all other districts, rather than by the lack of standard microbiome analyses as suggested by Hooks et al.

Independent from the applied technique, most studies remain at an observational level, and our understanding of the relationship between complex disorders and abnormalities detected in microbiota and derived microbial metabolites is still mostly speculative.

Neurotropic enteroviruses co-opt "fair-weather-friend" commensal gut microbiota to drive host infection and central nervous system disturbances

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Abstract

Some neurotropic enteroviruses hijack Trojan horse/raft commensal gut bacteria to render devastating biomimicking cryptic attacks on human/animal hosts. Such virus-microbe interactions manipulate hosts' gut-brain axes with accompanying infection-cycle-optimizing central nervous system (CNS) disturbances, including severe neurodevelopmental, neuromotor, and neuropsychiatric conditions. Co-opted bacteria thus indirectly influence host health, development, behavior, and mind as possible "fair-weather-friend" symbionts, switching from commensal to context-dependent pathogen-like strategies benefiting gut-bacteria fitness.

Hooks et al. critique top-cited peer-reviewed scientific literature in which authors claim to confirm that intestinal microbiota robustly manipulate the brains, behaviors, and minds of infected humans and animals. Perhaps epitomized by science review and sensational news media accounts of so-called mind-controlling *Toxoplasma* (Mayer et al. 2014; Sampson & Mazmanian 2015; Stilling et al. 2016), these reports and labels sometimes create disproportionate science and lay community excitement about the possible role played by the gut-brain axis in modifying host cognitive-emotional states, particularly eco-evolutionary explanations for neurodisease etiologies, symptoms, and prophylaxes/treatments (Liu 2017; Mayer et al. 2014; Sampson & Mazmanian 2015; Stilling et al. 2016). Hooks et al. disapprove of such trends and prescriptively warn against accepting popular experimental findings and interpretations of host-microbe symbiosis often justified with outdated, inconclusive, and/or unsound microbiome, neuroendocrine, behavioral, and statistical procedures. The authors' scrutiny of microbiota-gut-brain research should be welcomed by scientist and journalist alike and might help establish guiding principles for improving empirical approaches, as well as primary- and secondary-source reporting practices. However, in preferentially selecting a limited, albeit manageable, number of highly cited microbiota-gut-brain studies, Hooks et al.'s own critical assessment ironically under-represents the scientific richness and excellence found across a broader cross section of published research, including investigations verifying that harbored gut microbiota significantly affect the nervous systems and goal-directed behaviors of coevolved host organisms (cf. Clark 2013a; 2013b; 2018; Clark and Eisenstein 2013; Clark et al. 2013). The authors' inattention to that larger body of work, despite hopeful concluding statements about the basic and clinical science benefits of emerging microbiology research rigor, needlessly undermines their intent for fair accurate meta-analytical evaluation and meaningful fact-motivated advances within the field of inquiry.

To contrast and embellish Hooks et al.'s position based on 25 narrowly chosen literature examples, I enlist a small set of less cited peer-reviewed publications that typify capably performed and reported science demonstrating causal relationships between gut-brain-axis status and infected host health, development, behavior, and mind (cf. Erickson et al. 2018; Karst 2016; Kuss et al. 2011; Robinson & Pfeiffer 2014; Wilks & Golovkina 2012). These decade-spanning findings, proving neurotropic enteroviruses co-opt commensal gut microbiota during complex infection-cycle interactions, permit credible eco-evolutionary interpretations about infectivity, raising surprising doubt as to proper usage of "commensal" when defining benign symbiosis between various intestinal bacteria and host organisms. Through convergent evolution (e.g., zoonosis/zooanthroponosis) and/or coevolution pathways, certain neurotropic enteroviruses, such as RNA poliovirus, and their bacterial associates, such as nonpathogenic *Escherichia coli*, adapted a powerful biomimicry attack on human and animal hosts. Viruses, in such scenarios, increase their fitness by cryptically binding to bacteria-surface polysaccharides, a process termed "Trojan horse" or "raft" host incursion, to facilitate virus capsid/viron stabilization, host-immunodefense evasion, host-cell docking, and later (virus-virus) genetic recombination, replication, pathogenesis, and transmission. These cosmopolitan virus-microbe relations, ignored by Hooks et al. as legitimate mechanisms for gut-microbiota host manipulation, produce notable host central nervous system (CNS) disturbances at cellular and systems levels, including up-regulated cytokine and chemokine synthesis, apotosis and autophagy, neuroinflammation, and additional sequalae. Both immediate and delayed CNS disturbances may cause severe to fatal neurodevelopmental, neuromotor, and neuropsychiatric complications that further optimize virus fitness. Contrary to observed direct virus action, bacterial Trojan horses or rafts indirectly exert great harm over host health, development, behavior, and mind – prohibiting their classification as genuine commensal symbionts. Yet, the eco-evolutionary advantages of host-disease incidence, severity, timing, and duration remain poorly understood for the optimization of bacteria fitness and therefore continue to limit rational data interpretations.

Nevertheless, after reading Hooks et al.'s reproach of widespread unscholarly data interpretations in microbiota-gut-brain literature, one may expect bad reporting habits for the above-mentioned model of intestinal bacteria activity, similar to misleading mind-control attributions for *Toxoplasma* infections. Many playful, flashy, even personifying interpretations and labels, now common to modern-day multimedia presentation styles, enrich the public's imagery of difficult-to-comprehend science concepts. For example, the catchphrase of "fair-weather-friend" colorfully connotes still largely unknown bacterial benefits of switching from commensal to pathogen-like host-microbe symbiosis strategies. Such language, as noted by Hooks et al., frequently exceeds boundaries of sound scientific convention, exchanging masterful science for masterful hyperbole. Evidence of this tendency seems almost absent for reports on enterovirus-hijacked commensal gut bacteria. Inexhaustive searches of primary and secondary science-reporting sources yield few marginal descriptions, including "foe-or-friend" gut microbiota (David 2011), rightly challenging and qualifying eco-evolutionary notions of bacterial commensalism – a result that juxtaposes over-use of coined "viral understudy" and "friendly" to depict speculative host-promoting viral phenomena (e.g., Bordon 2015; Ray 2015). The reasons underlying competent interpretation and label use likely originate with responsible primary-science methods and reporting, which, in turn, help deter subsequent mischaracterization of facts in secondary sources, procedures well advocated by Hooks et al. If true, the majority of lesser cited microbiota-gut-brain articles, as compared with top-cited ones, might better comply with Hooks

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et al.'s recommendations for elevating science methods and journalism standards. Only more thorough literature-inclusive meta-analyses, waiting to be conducted by Hooks et al. and/or alternative authors, may thus validate the actual full impact of rarely to highly cited publications on the microbiota-gut-brain research field and the content of its science review and trendy news media narratives.

Hooks et al. employ literature meta-analysis to identify flaws in microbiota-gut-brain research. They ably craft a taut message around their findings, which emphasize the catastrophic consequences and potential remedies of accepting inferior work into the official scientific record, including empirically unfounded eco-evolutionary interpretations and buzzwords. The authors show indisputable good judgment in their call to science and lay communities for stringent research and publishing regulation. However, if literature meta-analyses become a fundamental tool to achieve those goals, as I believe they should, then assumptions supporting that effort must be consistent and complete. Unfortunately, the authors, like many people, mistakenly over-value highly cited peer-reviewed publications as representative, if not quality, science and miss superior research falling below arbitrary thresholds of professional and/or amateur popularity. Although Hooks et al. present microbiota-gut-brain research problems worthy of notice, their choice in science articles might not correctly define the microbiota-gut-brain field of study. This lingering confusion further reinforces growing desires to also perfect meta-analytical science review publishing standards with impartial, statistically argued comprehensive findings.

Nourishing the gut microbiota: The potential of prebiotics in microbiota-gut-brain axis research

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Abstract

Dietary fiber and prebiotics consistently modulate microbiota composition and function and hence may constitute a powerful tool in microbiota-gut-brain axis research. However, this is largely ignored in Hooks et al.'s analysis, which highlights the limitations of probiotics in establishing microbiome-mediated effects on neurobehavioral functioning and neglects discussing the potential of prebiotics in warranting the microbiota's role in such effects.

Central to Hooks et al.'s analysis is their critique of unwarranted causal claims about the impact of the gut microbiota on psychobiological functioning following probiotic interventions, given the inconsistent evidence on the capacity of probiotics to modulate gut microbiota composition and function. Although we agree, evidence on the effects of dietary fiber (DF) and prebiotics on microbiota composition and function and subsequent psychobiological changes are not discussed. We argue that such findings illustrate the potential of prebiotic interventions in supporting causal claims about the impact of gut microbiota on psychobiological functioning. Nonetheless, before meriting such claims, direct investigation into the mechanisms that mediate DF/prebiotic effects on brain function are needed.

Critical to warranting causal claims in microbiota-gut-brain (MGB) axis research is the availability of tools to steer the microbial ecosystem into a desired composition/function that subsequently improves brain function. DF/prebiotics may constitute such a tool. DF is defined as carbohydrate polymers with three or more monomeric units, which are not hydrolyzed by the endogenous small intestinal human enzymes, are naturally occurring or isolated from foods, and demonstrate a physiological health benefit (Jones 2014). Fermentable fibers provide metabolic substrates for most gut bacteria, influence their diversity and richness, and increase levels of fermentation products such as short chain fatty acids (SCFAs) (den Besten et al. 2013). Some fibers can be classified as "prebiotic" if they are "selectively utilized by host microorganisms conferring a health benefit" (Gibson et al. 2017, p. 493).

A systematic review and meta-analysis of 64 intervention studies in healthy adults found that DF resulted in consistently higher abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. (So et al. 2018). However, the application of next-generation sequencing techniques that allow microbiota-wide assessment of relative abundance shifts suggests that modification of microbiota composition by means of DF/prebiotics is not limited to these specific taxa (Davis et al. 2011; Everard et al. 2011; 2014; Holscher et al. 2015; Martínez et al. 2010; Vandeputte et al. 2017a; Walker et al. 2011). Notably, these studies indicate that changes in gut microbiota composition are reversible, maintained only with continued consumption of DF/prebiotics, and exhibit inter-individual variation probably dependent on baseline microbiota profile, including presence of keystone species or variation in enzymatic capacity of certain strains (Falony et al. 2016; Ze et al. 2013).

Although DF/prebiotics consistently modify gut microbiota composition, only a few studies have explored their effect on neurobehavioral functioning. Human studies, although notably scarce, showed positive effects on hypothalamic-pituitary-adrenal axis activity, emotional attention, and anxiety and depression symptomology (Azpiroz et al. 2017; Farhangi et al. 2017; Schmidt et al. 2015). Animal studies revealed effects on stress response (Forsatkar et al. 2017), anxiety- and depressive-like behavior (Mika et al. 2017; 2018; Savignac et al. 2016), stress-induced sleep alterations (Thompson et al. 2016), cognition

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(Gronier et al. 2018), and related neurobiological mechanisms such as gamma aminobutyric acid (GABA) and serotonin receptor gene expression (Burokas et al. 2017) and brain-derived neurotrophic factor (BDNF) and N-methyl-D-aspartate receptor subunit levels (Savignac et al. 2013; Williams et al. 2016). None of the human studies, and only some of these animal studies, concurrently quantified microbiota composition and found increased abundance in fecal *Bifidobacterium* and *Lactobacillus* spp. using selective bacterial culture or quantitative polymerase chain reaction (Azpiroz et al. 2017; Gronier et al. 2018; Kao et al. 2018; Mika et al. 2017; 2018; Savignac et al. 2013; 2016; Thompson et al. 2016). One study (Burokas et al. 2017) used 16S rRNA sequencing and showed changes in β-diversity and shifts at different taxonomic levels. A limited subset of these studies, exclusively in rats, correlated prebiotic-induced changes in microbiota composition and relative abundance with changes in brain function. Prebiotic-induced increases in fecal *Lactobaccillus* spp. positively correlated with altered cfos and serotonin receptor gene expression in multiple brain regions (Mika et al. 2018) and predicted stress-protective alternations in mRNA expression in serotonergic dorsal raphe nucleus neurons during inescapable stress (Mika et al. 2017). Furthermore, lower levels of Deferribacteres following a prebiotic diet correlated with longer non–rapid eye movement episode durations (Thompson et al. 2016). Following ingestion of fructo- or galacto-oligosaccarides, the number of fecal bifidobacteria correlated positively with frontal cortex NR1 protein (Savignac et al. 2013).

Bacterial fermentation of DF leads to the production of SCFAs (den Besten et al. 2013). SCFAs – predominantly acetate, propionate, and butyrate – constitute the major anions in the colon and serve as an energy source for colonocytes. Furthermore, they inhibit histone deacetylation and activate G-protein coupled receptors, thereby acting as signaling molecules linking diet, gut microbiota, and host (Tan et al. 2014) and interacting with gut-brain signaling pathways (Dalile et al. 2019). Few studies have explored whether the effects of DF/prebiotics on brain function are mediated by SCFAs. Fructo- and galacto-oligosaccaride-induced increases in cecal SCFAs correlated with effects on depressive and anxious behavior and stress responses, as well as changes in gene expression in mice (Burokas et al. 2017). Prebiotic Bimuno galacto-oligosaccharides increased plasma acetate levels (Gronier et al. 2018; Kao et al. 2018), cortical GluN2B subunits (involved in glutamate neurotransmission), and acetyl-CoA carboxylase mRNA, all of which also increased following direct administration of acetate (Gronier et al. 2018), suggesting that acetate may play a mechanistic role in the observed effects. Other studies that explored the effects of SCFA administration on brain function have been reviewed elsewhere (Dalile et al. 2019).

Although current evidence does not convincingly support a causal role of gut microbiota in modulating neurobehavioral functioning, we believe that prebiotics have a higher potential than probiotics to warrant such causal claims. However, before maintaining such claims, high-quality, adequately powered (human) prebiotic intervention studies, measuring both changes in microbiota composition or function (Bindels et al. 2015) and psychobiological functioning using state-of-the-art methodology, are needed. Such interventions should use mediation analysis to estimate the contribution of microbiota composition/function in the observed psychobiological effects. Claims that causally implicate the role of gut microbiota in the MGB axis should be based on studies that isolate products of microbial activity and directly demonstrate their causal effects on the brain.

Notes

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2. Authors Kristin Verbeke, Lukas Van Oudenhove, and Bram Vervliet contributed equally to this work as senior authors.

Neuropeptide-like signaling in the microbiota-gut-brain axis

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Abstract

For gut microbiota to influence behavior, microorganisms should be able to interfere with specific brain neurochemical circuitries. Understanding these molecular mechanisms is a key task in the new microbiota-gut-brain field. Recent studies have revealed that one major mechanistic link is the modulation of neuropeptide signaling by homologous bacterial proteins acting both directly and indirectly via production of neuropeptide-reactive immunoglobulins.

The associations between dysbiotic microbiota and various physiological and pathological brain states and behavior have been reported, including some most highly cited papers selected for critical analysis by Hooks et al. Multiple problems of the new field of microbiota-gut-brain (MGB) have been rightly highlighted including the current lack of causality between dysbiosis and behavior. As the solution to this problem and for future directions the authors suggest focusing on "more connected research … in multipathway systems" (sect. 9, para. 1). I find this suggestion too vague and would instead propose to focus the MGB research on determining the possible role of specific bacteria in modulating brain function and behavior. Moreover, because the MGB research is connecting with the mature scientific fields of neuroscience, neuroendocrinology, and neuroimmunology, it could be more constructive to design new MGB studies that target the well-known neurochemical systems. The ultimate goal of such studies would be identification of molecular tools used by specific gut bacteria to influence the specific brain pathways including molecular and cellular targets. Classic neurotransmitters, such as GABA, dopamine, and so forth, and neuropeptides may be among the primary targets of microbiota interference. My further comment gives an example of experimental and conceptual approaches to promote the specificity in MGB research.

Neuropeptides and peptide hormones may participate in the MGB signaling because of their specific roles in the regulation of various brain functions and behavior (Hökfelt et al. 2003). The specificity of each neuropeptide is determined by its unique amino acid sequence binding with a nanomolar affinity to neuropeptide receptors. These properties of neuropeptides make them

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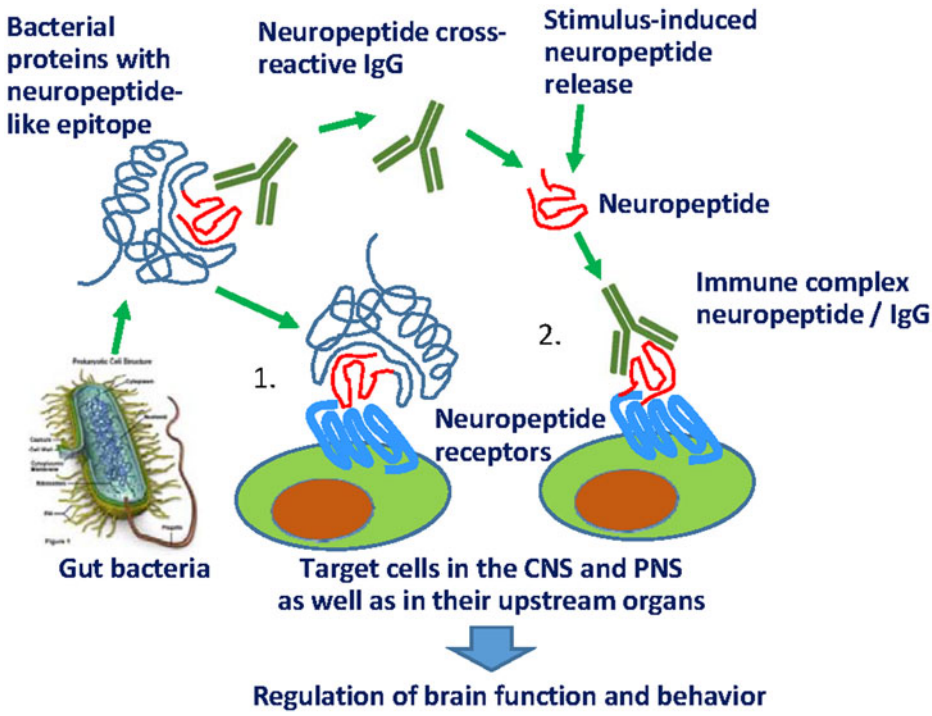


Figure 1 (Fetissov). Simplified model illustrating the concept of microbiota influence of peptidergic signaling in MGB axis: (1) Binding of bacterial mimetic proteins to neuropeptide receptors may directly modify peptidergic signaling. (2) Bacterial mimetic proteins may trigger production of neuropeptide cross-reactive IgG forming functional immune complexes with neuropeptides providing an indirect mechanism of microbiota influence on peptidergic signaling. This model supposes either stimulation or inhibition of neuropeptide signaling in central and peripheral nervous systems (CNS and PNS) depending on the molecular properties of bacterial mimetic proteins and immune complexes, effectively linking microbiota composition with neuropeptidergic regulation of brain function and behavior.

perfect targets for microbiota-derived homologous proteins. Indeed, this type of MGB mechanism of communication was recently described for α-melanocyte-stimulating hormone (α-MSH), a neuropeptide of the melanocortin (MC) family involved in regulation of energy metabolism, memory, and emotion. A heat shock protein, ClpB, which plays a key role in protein disaggregation in bacteria (Mogk 2003), was found to contain an α-MSH-like motif (Tennoune et al. 2014). A complementarity of this motif towards the MC receptors was then demonstrated (Ericson et al. 2015) providing the mechanistic background for a direct α-MSH-like effect of ClpB. In support of such molecular mimicry, mice receiving native *Escherichia coli*, but not ClpB-deficient bacteria, displayed reduced food intake and body weight (i.e., demonstrating an α-MSH-like anorexigenic effect of ClpB [Tennoune et al. 2014]). Moreover, ClpB activated the arcuate nucleus proopiomelanocortin neurons, a key structure in the brain anorexigenic pathway (Breton et al. 2016b). Finally, to reach the brain ClpB should be present in blood, and in fact, it was detected in plasma of healthy humans (Breton et al. 2016a), while its plasma concentration in rats was proportional to the ClpB DNA expression in feces (Breton 2016b). It is also of importance for the concept of MGB signaling to mention that the α-MSH-like motif of ClpB was specific to the ClpB protein expressed by the family of Enterobacteriaceae (Fetissov et al. 2019). Hence, the ClpB protein appears as an endocrine neuropeptide-like factor, whose production is regulated by gut bacteria (i.e., dependent on gut microbiota composition).

This discovery of the α-MSH-like properties of ClpB was initially triggered by the observation of α-MSH-reactive autoantibodies in humans and rodents. Subsequently, ClpB has been found to act as an antigen mimetic of α-MSH – that is, active immunization or *per os* provision of ClpB-expressing *E.coli* bacteria leads to increased plasma level of anti-ClpB α-MSH cross-reactive immunoglobulins (Ig's) (Tennoune et al. 2014). The relevance of such α-MSH-reactive IgG and IgM to brain function and behavior was shown by significant correlations of their plasma levels with psychopathological scores in patients with psychiatric disorders including anorexia nervosa and bulimia (Fetissov et al. 2005), as well as with depression and anxiety scores in healthy subjects (Karaiskos et al. 2010). Further molecular insight comes from the finding that α-MSH in the circulation forms immune complexes with IgG that activate the MC4 receptors with a lower threshold than α-MSH alone. Importantly, different IgG α-MSH binding epitopes in patients with obesity and anorexia nervosa were accompanied by altered kinetics of immune complex formation and MC4 receptor binding and activation. Such modulation of MC4 receptor signaling may contribute to altered regulation of appetite and energy metabolism in patients (Lucas et al. 2019). Considering the causal role of specific bacterial antigens in production of α-MSH-reactive IgG, it is likely that the altered levels and binding properties of α-MSH-reactive IgG in patients with obesity and eating disorders may result from altered antigenic composition of their gut microbiota.

Taken together, the ClpB/α-MSH homology provides an example of a specific molecular link between gut bacteria of the Enterobacteriaceae family and the MC system regulating feeding behavior. Such data are valuable for the interpretation of the potential impact of gut microbiota composition on the MGB axis. For example, when increased abundance of Enterobacteriaceae is detected, over-activation of the MC system, including low appetite, can be expected. This example can also be generalized as a concept for neuropeptide-like signaling in the MGB axis applicable to other neuropeptides and peptide hormones (Fig. 1). In fact, healthy humans possess plasmatic immunoglobulins reactive with several key regulatory peptides displaying sequence homology with proteins derived from gut microbiota (Fetissov et al. 2008). For example, natural IgGs reactive with corticotropin were shown to interfere with cortisol secretion and aggressive behavior (Værøy et al. 2018), ghrelin-reactive IgGs were found to improve ghrelin stability and to enhance feeding behavior (Takagi et al. 2013), whereas oxytocin-, vasopressin-, ghrelin-, and neuropeptide Y (NPY)-reactive natural IgGs were associated with anxiety and

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depression (François et al. 2015; Garcia et al. 2011; 2012). It is, hence, likely that the production of such neuropeptide- and peptide hormone-reactive IgG may be directly linked to the homologous antigenic stimulation from gut microbiota. Discovery of neuropeptide-like antigenic bacterial proteins should represent then one of the main tasks for improving our understanding of the functionality of the MGB axis at least as it concerns the specific peptidergic systems regulating brain functions and behavior.

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Scientific claims are constitutive of common sense about health

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Abstract

Endorsing the view that commonsense conceptions are shaped by scientific claims provides an explanation for why microbiota-gut-brain (MGB) research might become incorporated into commonsense notions of health. But scientific claims also shape notions of personal identity, which accounts for why they can become entrenched in common sense even after they have been refuted by science.

At the end of their critical analysis of microbiota-gut-brain (MGB) research, Hooks et al. identify the potentially negative consequences of the popularity of this research in the press and on social media. They aptly point out that the appeal of some of these studies is their continuity with what they characterize as commonsense conceptions of health. In this commentary, I will briefly describe a conception of common sense that can be used to provide an explanation for the easy absorption of MGB research into commonsense views about health and why this might be detrimental.

Commonsense conceptions tend to be characterized in contrast to scientific notion (i.e., common sense is not science, and commonsense concepts are not scientific concepts). When they are considered unscientific, commonsense notions are often described as intransigent or static, which can discourage attempts to change them. This way of characterizing common sense is wrong because it ignores the ways in which it is shaped by science. A better way of defining common sense is as continuous with science (Sellars, 1963). Even further, the right characterization of commonsense views is as empirically evaluable folk theories, such as folk psychology (Churchland, 1992) or folk morality (Gligorov 2016) used in everyday life to explain and predict human behavior. As I have argued previously (Gligorov 2016), folk conceptions are influenced by scientific discoveries and are in fact shaped by them in a variety of different ways. Advances in neuroscience have promoted the identification of psychological traits with brain processes and have shaped our conceptions of personal identity, privacy, free will, and even our notions of death (Gligorov 2016).

Similarly, the human microbiome might shape commonsense conceptions of health and of personal identity (Gligorov et al. 2013). This might happen by characterizing individuals as superorganisms and shifting conceptions of health to include keeping a healthy microbiome. Furthermore, being a superorganism might expand moral responsibilities to include the duty to keep a healthy microbiome and prevent the spread of disease (Battin et al. 2008). Incorporating facts about the human microbiome would lead to a reconceptualization of what it is to be a healthy person or what is required to lead a healthy lifestyle. For example, claims that certain bacteria will promote health might change attitudes away from preventing contamination. The rush to use probiotics is one way in which this change in attitude is manifested. MGB research would have an even more straightforward effect on views about personal identity because the claim is that changes in gut microbiota can affect psychological traits, such as anxiety or mood, which are constitutive of how we think of ourselves.

The potential shifts in common sense that might occur because of MGB research would be only the latest iteration of science shaping common sense. Consider that any of the claims cited by Hooks et al. as being part of our common sense about health and nutrition, such as eating fresh food, minimizing fat and sugar, and more exercise are all edicts based on scientific studies establishing causal connections between nutritional styles and some aspect of our health. But because most of these recommendations are learned secondhand by the public, not from scientists or scientific publications, their scientific etiology can be lost.

Circling back to the worries raised by Hooks et al., one is that MGB research is influencing everyday notions of health prematurely and promoting perhaps false beliefs about how to treat psychiatric disease, and the other is that MGB research seems to be confirming commonsense platitudes about health rather than adding to them in ways that might lead people to forego taking medication when they might need it. I would like to underscore both of these worries by using the view I just described about how MGB research can change personal identity.

If it is the case, as I argue, that commonsense notions are not only affected, but also continuously revised by scientific discoveries, then the popularization of MGB research will have an impact on common sense about health. Whether that influence is positive or negative will trail the quality of MGB research and the accuracy of the claims derived from that research. But there is one way in which early scientific mistakes could have more permanent and perhaps negative consequences on common sense. Although science is self-correcting because there are established ways in which unsupported claims can be eliminated, the path to self-correction is not as well trotted in common sense. One of the reasons for why commonsense notions about health might be particularly difficult to revise is precisely because they become incorporated into conceptions of identity. Once an individual becomes committed to being healthy in particular way, then that becomes a part of their narrative identity – it becomes part of how they tell a story of who they are (DeGrazia 2005). Additionally, whether they keep healthy and live well takes on moral

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dimensions and becomes action guiding; they wish to continue living the right way. This is why scientific claims that are continuous with beliefs already endorsed, say about what is natural or healthy, are much easier to reinforce than they are to revise. For example, if individuals become committed to living a natural and healthy lifestyle, which they think requires cultivating the microbial environment in their gut buy using probiotics, any study that confirms that particular way of keeping healthy will be easier to believe because it is already congruent with their established concept of health. Similarly, scientific claims challenging established views about health will take longer to become entrenched in common sense because they require changes not only to particular beliefs, but also to parts of an individual's narrative identity. Hence, beliefs about the causal connections between gut microbiota and brain health, if disproved, might be easier to revise within the relevant scientific community than they might be to eliminate from common sense.

Putting microbiota-gut-brain research in a systemic developmental context: Focus on breast milk

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Abstract

The microbiota-gut-brain (MGB) field holds huge potential for understanding behavioral development and informing effective early interventions for psychological health. To realize this potential, factors that shape the MGB axis in infancy (i.e., breast milk) must be integrated into a systemic framework that considers salient behavioral outcomes. This is best accomplished applying network analyses in large prospective, longitudinal investigations in humans.

Microbiota-gut-brain (MGB) research suggests that early bidirectional interactions of the gut-brain axis may have important and long-lasting effects on physical and psychological development. Early in life, the brain and the gut microbiome undergo dramatic parallel structural and functional changes. Work in understanding this co-development is paving the way to a developmental framework of brain-behavior connections that includes metabolic processes beyond the central nervous system. Although Hooks et al. provide a brief discussion of the development of gut-brain connections, the view that they present is shallow and leaves the impression that examinations of the impacts of microbial changes on brain and behavioral development are not promising. Important findings such as the dramatic developmental changes of germ-free animals (Luczynski et al. 2016) and the effect of fecal transplantation on behavior (De Palma et al. 2017; Kelly et al. 2016) provide crucial evidence that the microbiome and brain are parts of a system of interactions that promotes healthy physical and behavioral development. As Hooks et al. highlight, the majority of this evidence comes from rodent studies, and obvious difficulties exist regarding both extrapolation and isolation of causal effects in humans. However, disregarding these works and overly downplaying promising achievements of MGB research would be a mistake.

Hooks et al. present a timely reality check for all MGB researchers, as they warn against hyping the importance of single experiments to conclude that the microbiome is directly and univocally causally responsible for healthy physical and behavioral outcomes. Warding off this risk, the challenge remains to embrace a comprehensive developmental framework that can uncover the conditional role of various components in behavioral development, such as nutrition and the microbiome as important environmental factors. Early nutrition (i.e., breast milk) is one important factor linked to infant gut microbiome composition (Bäckhed et al. 2015; Planer et al. 2016) and possibly to brain development (Isaacs et al. 2010). Furthermore, neurons in developing brains express receptors for microbial products more extensively early in development (prior to weaning) than later in adulthood (Arentsen et al. 2017), which suggests a plausible mechanism for gut-brain communication in infancy and also suggests the existence of a sensitive period marked by consumption of milk. As milk is the sole source of nutrition for most infants, it has huge potential to study the effect of nutrition on the microbiome early in life. Recent evidence suggests that differences in early diet and microbial exposure have effects on microbiome establishment and maturation (Diaz Heijtz 2016). For example, length of breastfeeding contributes to shifting the timing of maturational changes in microbial community structure (Bäckhed et al. 2015). Additionally, components of human milk, specifically the milk microbiome and human milk oligosaccharides (HMOs) appear to be crucial in establishing and maintaining the gut microbiome (Jost et al. 2015; Pannaraj et al. 2017). Interestingly, both microbiome composition (Christian et al. 2015) and milk factors (Grey et al. 2013; Nolvi et al. 2018) have been linked to fear and anxiety behaviors; however, no studies to date have looked at these in a single integrated framework.

The majority of developmental MGB work has looked at simple connections between either environmental factors and the microbiome, the microbiome and brain measures, or the microbiome and behavior, whereas very few have looked at modulators of the microbiome, neurodevelopment, and behavioral outcomes in a single cohort (Carlson et al. 2018). It is vital that ongoing and future work identifies relationships among these diet and environment-linked changes in microbial community structure and concurrently measured behavior and brain structure and function. This requires that MGB research be implemented comparatively and longitudinally on humans using large cohorts of subjects while applying robust statistical strategies that are able to highlight network dynamics. Employing advanced data analytic approaches (Kelsey et al. 2019; Xia & Sun 2017) to integrate microbiome compositional patterns that are different among different outcome groups – for example, high and low performers on a behavioral task (as opposed to grouping the microbiome and looking for performance differences between the groups as was done in Carlson et al. [2018]) – would improve our understanding of these relationships and would provide a developmental and behavioral context in which to make determinations regarding what an optimal microbiome actually is.

In this way, we may be able to highlight how and when interindividual variability in microbiome composition reflects variability in behavioral development. Interestingly, Carlson and

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colleagues used this approach and highlighted that breastfeeding at the time of sample collection was one of the most robustly predictive covariates for the identification of clusters reflecting possible cognitive differences. If confirmed, studies like this will pave the way to non-invasive therapies based on supplementation of diet (in lactating mothers, as well as infants themselves) for optimizing brain and behavior development early in life when interventions are thought to be most impactful. In conclusion, our message is that considering the importance of infancy for the development of MGB interactions, and that early experiences, such as breastfeeding, can shift developmental trajectories, there are huge implications for integration of diet, as milk is the primary form of nutrition and is dynamic. If we want to be able to leverage the microbiome to optimize behavioral and neurodevelopmental outcomes, we must understand not only how the microbiome affects brain and behavioral development, but also how developmentally salient environmental factors affect the microbiome.

Why a developmental cognitive neuroscience approach may be key for future-proofing microbiota-gut-brain research

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Abstract

Here we argue that a multidisciplinary research approach, such as currently practised in the field of developmental cognitive neuroscience, is key to maintaining current momentum and to future-proof the field of microbiome-gut-brain research. Moreover, such a comprehensive approach will also bring us closer to our aims of translation and targeted intervention approaches to improve mental health and well-being.

The gut microbiome has recently emerged as an important new player in our efforts to understand the different factors that influence human behaviour (McVey Neufeld et al. 2016; Sarkar et al. 2016; Tang et al. 2014). Gut and brain are intimately connected via the gut-brain axis, which involves bidirectional communication via neural, endocrine, and immune pathways (Grenham et al. 2011; Grossman 1979; Mayer et al. 2014). Research in both human and animal models has also highlighted the important role that gut microbiota play in regulating the brain and subsequent behaviour, particularly within the context of mental health problems such as anxiety or depression (Cryan & Dinan 2012; Foster & McVey Neufeld 2013; Mayer 2011).

It is not surprising, therefore, that the microbiome, and its effect on behaviour and mental health, has captured the interest and imagination of scientists and the wider public alike. As a result, research in this relatively new area has intensified, as have funding opportunities that aim to close the many gaps in our understanding. The substantial translational potential of this research and the opportunities to establish links with industry may also play a significant role in this development. In their target article, Hooks et al. highlight the enormous potential that the new area of microbiota-gut-brain (MGB) axis research holds for understanding human behaviour, yet they also rightly point towards several concerns in research practice that are not necessarily specific to the field of MGB research but are of relevance to broader scientific practice in examining human behaviour. The greatest concern is that public expectations and commercial efforts have already overtaken the relatively small number of research publications to date, and there is a significant risk of backlash if the foundations of the young MGB field prove to be less than beneficial.

So how can the combination of human development and MGB research contribute to mental health research and interventions? It has been shown that a significant reduction in microbial diversity in the gut, known as dysbiosis, affects brain-behaviour relationships and may lead to psychological abnormalities, as common in mental illness (Foster & McVey Neufeld 2013). In adults, for example, dysbiosis has been shown to be related to symptoms of anxiety and depression (Mayer 2011; Mayer et al. 2014). Most importantly, animal research has repeatedly shown that the *timing* of dysbiosis is important, and that the period of adolescence may be a critical window during development where microbiota help fine-tune the gut-brain axis related to stress responses and anxiety (Foster & McVey Neufeld 2013). This suggests that the consequences of dysbiosis will be particularly critical during development, as ongoing maturation and increased plasticity levels can lead to atypical behavioural patterns and brain network maturation (Cohen Kadosh & Johnson 2007; Cohen Kadosh et al. 2013). The psychiatric literature supports this hypothesis: Age-of-onset data show that first symptoms of many psychiatric disorders, including (social) anxiety or depression emerge at the adolescent juncture (Keshavan et al. 2014; Kessler et al. 2005). We therefore believe that MGB research could provide the missing link that brings together previous research in human brain development and mental health.

We believe that it is now important to take advantage of the promising findings in animal-model research to investigate whether similar effects of the timing and effects of dysbiosis also apply to the human model and to investigate the psychoactive properties of the microbiome in the transition from childhood to adulthood. Animal models suggests that one way of influencing microbial diversity and reversing dysbiosis is via nutrition, and it has been shown that drastic changes in diet can alter microbial diversity in mere days (David et al. 2014). This opens up new opportunities for both prevention and intervention, but more research is still needed to show how we can benefit from this window of plasticity to shape the developmental trajectories in at-risk groups.

Here, we would like to draw attention to the important contribution that developmental cognitive neuroscience (DCN) research can make for establishing causal relationships between dysbiosis and mental health problems during the critical developmental period when the gut-brain axis is fine-tuned and when atypical patterns will have long-lasting consequences. The DCN research approach focuses on investigating how the complex

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interplay of genetic, environmental, and brain maturational factors shape psychological functioning in development to improve the outcome for the individual (Cohen Kadosh 2011; Johnson et al. 2002; 2009). Moreover, placed at the intersection of nature versus nurture, the DCN research approach always assumes a multilevel and multifactor approach to understanding change, which, by definition, is multidisciplinary. Given that the field of microbiome and gut-brain axis research is still emerging and finding its shape, we are strongly convinced that any real progress will depend on the adoption of a similarly comprehensive multifactorial and multidisciplinary research approach for pinpointing mechanisms and translation in both animal and human models. Such an approach would also be able to account for specific critical periods in development, when change happens at many levels simultaneously. We therefore agree with the authors that discussion must be given to specifying mechanisms, differentiating correlational from causal explanations, and addressing a priori realistic outcomes. Moreover, there needs to be rigorous assessment in human populations, coupled with well-defined research questions and appropriate statistical analysis. This is particularly important given that strong public and commercial interests are presently outpacing research efforts. Based on an extensive body of research in the field of DCN, we are aware that even simple changes in behaviour or diet can have long-lasting effects on the brain, and mental health and well-being, all of which need to be considered as the ethical implications are significant. Therefore, to maintain current momentum and to future-proof the field of MGB research, a multidisciplinary research approach such as currently practised in the field of DCN is key if we want to reach our aims of translation and targeted intervention approaches to improve mental health and well-being.

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A call for mapping the development of the microbiota-gut-brain axis during human infancy

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Abstract

We argue for the importance of looking at the microbiota-gut-brain axis from a human developmental perspective. For this purpose, we first briefly highlight emerging research with infants attesting that the microbiome plays a role in early brain and cognitive development. We then discuss the use of developmentally informed humanized mouse models and implications of microbiome research that go beyond probiotic administration.

In the target article, Hooks et al. critically review the current state of microbiota-gut-brain axis research in animal models and make specific suggestions on how to improve research in this area. However, Hooks et al. appear to have overlooked what might be considered one of the most promising avenues for moving research in this emerging field forward. Specifically, we would like to argue that the time is ripe to explore the role of the human microbiota in brain and cognitive development, especially during infancy (Kelsey et al. 2019).

From birth to age 3, the gut microbiome changes from a relatively sterile environment to a diverse ecosystem with thousands of species of bacteria, suggesting that this might represent a formative, and possibly sensitive, period in microbiota-gut-brain axis development (Borre et al. 2014; Walker 2013). The target article highlights initial support from animal models (e.g., Sudo et al. 2004), showing that the timing of bacterial colonization plays an important role in the development of the gut-brain axis, yet it fails to acknowledge existing evidence from humans, which further supports the notion that early development during infancy may critically shape the microbiome-gut-brain axis. For example, both delivery and infant feeding methods, which have been shown to affect the gut microbiome composition in infants, have also been identified as risk factors for early emerging neurodevelopmental disorders such as autism spectrum disorder (Curran et al. 2015; Dominguez-Bello et al. 2010; Heikkilä & Saris 2003). These epidemiological findings provide indirect, correlational evidence for a microbiota-gut-brain axis link in early human development.

More direct evidence for such a link comes from a pioneering recent study by Carlson et al. (2018) in which fecal samples were collected from 89 typically developing infants and analyzed using 16S ribosomal RNA amplicon sequencing. In this study, the link between infant gut microbiome composition at 1 year of age and cognitive development (measured by the Mullen Scales of Early Learning) and brain development (measured using structural magnetic resonance imaging), at both 1 and 2 years of age were assessed. Carlson et al.'s (2018) analysis revealed that cognitive development scores differed significantly between infants assigned into one of three gut microbiome taxonomic groups, as identified by cluster analysis. This study also reports some specific structural brain differences linked to the microbiome composition. However, it should be noted that the majority of structural brain measures, such as intracranial volume, total white or gray matter, total cerebrospinal fluid, or lateral ventricle volume, did not reveal any differences between infants for the three bacterial composition groups. Moreover, from these data, it is still unclear how the small volume differences found in specific brain areas are related to infant brain and cognitive function. Contrary to what is known from adults where higher microbial diversity has typically been shown to be predictive of positive health outcomes (Abrahamsson et al. 2014; Kostic et al. 2015), Carlson et al. (2018) showed that increased microbial alpha diversity was associated with lower cognitive performance in infancy. Based on this discrepancy, Carlson et al. (2018) suggest that microbial diversity may affect cognitive functions differently in infancy than later in development. In any case, the study by Carlson et al. (2018) as a first of its kind sheds new light on how individual differences in brain and cognitive development during infancy emerge in the context of the developing microbiome-gut-brain axis. Collectively, this points to the importance of developmental research, which systematically maps associations between microbial characteristics and brain and cognitive development across the entire human life span.

Related to taking a human developmental perspective, another potentially overlooked research approach is underscoring the use of developmentally informed humanized mouse models in order

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to create more translatable research. In the target article, authors make a poignant argument that there are inherent issues when one tries to make inferences about human mental health disorders from studies with animal models. The authors suggest that this area of research often uses language that overextends the implications of germ-free mouse models and rodent behavioral tests to human mental health. However, they fail to mention an alternative methodological strategy to addressing the issue of translatability, which is by creating humanized mouse models (for a review, see Walsh et al. 2017). Specifically, fecal samples from humans can be taken from clinically relevant populations (with or without mental health issues) at different points during development (from newborns to aging populations) and transplanted into animals – thus creating developmentally informed animal models that allow for a more mechanistic study of the microbiome-gut-brain axis.

Finally, we would like to argue that the implications for research on the early development of the microbiome-gut-brain axis in humans extend well beyond the somewhat overemphasized field of probiotic research. Specifically, in the context of infant development, research in this field has potentially major implications for delivery and neonatal care procedures. For example, medical facilities have recently started to examine the health benefits of "seeding" procedures, whereby infants delivered via C-section are wiped with maternal vaginal swabs, with the hope of colonizing infants with more diverse groups of bacteria. Moreover, the benefits of breastfeeding on infant brain and cognitive development have been widely studied and documented (Krol & Grossmann 2018). However, the gut microbiome has been largely ignored as a potential contributor to the positive effects of breastfeeding on infant and child development. Therefore, recognizing the need for incorporating a microbiome perspective in delivery and breastfeeding research with infants might help inform clinical practice.

Taken together, this commentary is intended to emphasize the importance of looking at the microbiota-gut-brain axis from a human developmental perspective with a specific focus on infancy. In addition, this commentary is meant to encourage the use of humanized animal models to tackle translatability issues and realize implications of this work, which extend well beyond probiotic administration. Overall, the hope is to complement the target article by inspiring the bold research programs needed to systematically examine the microbiome's role in early human brain and cognitive development.

Why don't probiotics work?

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Abstract

The conclusions reached by Hooks et al. urge the field to investigate the complex multipathway interactions between the microbiome and the gut-brain axis to understand the potential causal relationships involved. Claims in the field of microbiota-gut-brain research remain problematic without appropriate controls and adequate statistical power. A crucial question that follows from the authors' extensive review is: "Why don't probiotics work?"

The extensive review by Hooks et al. provides a critical analysis of the many claims that are made about the relationships between gut, microbiota, and human behavior. The authors do not deny that the research field of microbiota-gut-brain (MGB) is very important, and neither do they reject the many amazing results from animal studies. The main conclusion they present, supported by convincing evidence, is the oversimplification and overselling of animal findings in MGB research and the suggested far-reaching implications for human mental health. Interestingly, and in accordance with Hooks et al., the European Food Safety Authority did not award any claim of probiotics on physical or mental health (European Commission 2016). We agree, that in the long term, overblown claims damage the scientific credibility of MGB research, and that this may contribute to the erosion of the public's trust in science. We would like to add another concern, namely, that of the reduced support of animal testing by the general public when false claims based on animal experiments cannot be replicated in human studies. Another problem that has also been addressed by Hooks et al. is the use of animal models of mental disorders, where healthy or germ-free animals are used, which do not explain or mimic pathological mechanisms in ill psychiatric patients. In these animal models, behavioral findings could easily have been explained in terms of coping mechanisms (Korte et al. 2005). In addition, stress hormones are not necessarily bad; they serve the process of healthy adaptation (Korte et al. 1996). At this moment, there is neither evidence that complex (developmental) mental disorders, like autism, schizophrenia, Parkinson's disease, depression, posttraumatic stress disorder (PTSD), or anorexia nervosa, are caused by "bad" bacteria or a "leaky gut," nor that these often chronic mental disorders can be cured by "good" bacteria. Alternative explanations of positive short-term actions of probiotics on brain and animal behavior are possible. For example, probiotics may be helpful in the clearance of gram-negative bacteria, such as *Escherichia coli* (Timmerman et al. 2004). These *E. coli* bacteria have lipopolysaccharides (LPS) in their outer membrane that can produce inflammation. It has been shown that this increased the number and biological activity of serotonin transporters (SERTs) expressed by neurons and astrocytes and thereby reduced extracellular serotonin concentrations in prefrontal cortex and nucleus accumbens (Korte-Bouws et al. 2018). In addition, LPS-induced anhedonia (i.e., inability to feel pleasure) was abolished in SERT-knockout animals, suggesting that inflammation produces depressive-like symptoms via increased SERT activity (van Heesch et al. 2013). We agree with Hooks et al. that in future MGB research appropriate positive and negative controls and adequate statistical power have to be included to allow for the identification of cause and effect relationships. In addition, the field of MGB research would greatly improve when more attempts are made to falsify the hypothesis (Popper 1963) that probiotics are always good. Successful examples of this approach are shown in recent studies. In healthy volunteers, it was shown that probiotics could not successfully colonize the gastrointestinal (GI) tracts of all

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participants, but only succeeded to do so in some (Zmora et al. 2018). After a treatment with antibiotics, the same probiotics colonized the GI tracts of all test participants, but surprisingly, this colonization prevented the host's normal microbiome and gut gene expression profile from returning to its own original state for months afterward (Suez et al. 2018). However, when autologous fecal microbiome transplant (aFMT) was used (i.e., the host's own bacteria collected from before the use of antibiotics), the native gut microbiome returned to normal within days. Hence, probiotics are not a "one-fits-all" solution; a personalized approach is necessary. Furthermore, attention should be given to the role of the native gut microbiome, the disturbance of it at a young age, and the possible long-term consequences for health. The influence of prebiotics on the recovery of the native gut microbiome deserves further attention. The abovementioned findings clearly show why results obtained with probiotics in MGB animal research cannot always be directly translated into successful human applications. In summary, we fully agree with Hooks et al. that MGB research is a field full of promise, but, indeed, only when in the future putative causal relationships into the complex multipathway interactions between the microbiome and the gut-brain axis are investigated and a more critical approach is adopted.

Why microbes, not microbiomes, are better causal explanations in gut-brain research

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Abstract

Much microbiota-gut-brain research focuses on the causal role of microbiomes as a whole, rather than their component parts: microbes. Hooks et al. find these whole-community explanations inadequate; however, they do not provide suggestions for better explanations. By appealing to proportionality – a criterion that can be used to develop more appropriate causal explanations – more accurate causal claims can be made.

Hooks et al. identified many important problems in the methodology and interpretations of microbiota-gut-brain (MGB) research. Here I focus on one facet of MGB interpretations: the tendency to explain experimental effects by appealing to the microbiome as a whole. The authors allude to the problem of citing whole microbiomes – entire ecosystems or communities of microbes – as causes of behaviour and brain states. Yet a positive account of more appropriate explanations for these findings is not offered. I propose that this explanatory inadequacy is attributable to a failure of proportionality of explanation, given the current state of research in the field.

Proportionality is an explanatory tool that philosophers of science use to identify the most appropriate causal explanation when assessing scientific findings. Proportional explanations must not be too broad that they include aspects that are irrelevant to the effect produced, and not so narrow that important causal information is omitted (Woodward 2010). For example, if a pigeon is trained to peck whenever a red target is presented, then a disproportionate explanation for pecking behaviour would be the presentation of a coloured target. Citing a coloured target as a cause is too broad as it encompasses factors irrelevant to the effect, namely, other (non-red) coloured targets. Explaining pecking behaviour as caused by the presentation of a scarlet target is too narrow, as it excludes other variations of red that would also elicit this behaviour. A proportional explanation for pecking behaviour is simply the presentation of a red target (Yablo 1992).

In MGB research, microbiomes are cited as causes in one of two ways. The first is explicit reference to the whole microbial community as causing a brain or behavioural state (for examples, see sect. 7.1, para. 2). The second is when the microbiome as a community is implicitly suggested by reference to "dysbiosis" – a concept that indicates that the microbiome is "imbalanced," often implying decreased microbial diversity (for examples, see sect. 7.1, para. 3). To convincingly explain brain or behavioural states with microbiomes, the whole microbiome must be shown to be causally relevant. Researchers must demonstrate that manipulating microbiomes as a whole produces effects that cannot be reduced to just some of their component parts – via manipulations of one or a few microbes alone.

Most of the evidence presented regarding MGB research, however, does not support the thesis that the entire microbiome is causally involved. Manipulations using probiotics (Supplementary Table 2 in Hooks et al.) introduce one or at most a few species of bacteria – a tiny component part of the microbiome in its entirety. Making causal claims about microbiomes in these instances is akin to claiming that a coloured target caused the pigeon to peck. The implication is that entire communities of microbes exert a causal influence on behaviour, yet experimental evidence suggests single or a cluster of few microbes as proportional causal explanations.

Some researchers object to reductionist explanations of this kind by claiming that interventions using one or a few strains of bacteria make significant changes to the microbial community (e.g., Arnold et al. 2016). However, the burden of proof is on researchers to demonstrate (1) these large-scale changes occur after single species introductions, and (2) such changes are responsible for the brain or behavioural state observed, as opposed to the microbial introductions acting directly. Supporting (1) and (2) seems unlikely, as a review of probiotic treatments on human microbiota revealed no overall effects on α-diversity, richness, or evenness (Kristensen et al. 2016).

Probiotics are not the only interventions made in MGB research. Other methods include the use of antibiotics to "deplete" the microbiome and transplanting faecal matter from humans or other rodents into germ-free rodents. It could be argued that these more strongly support whole-microbiome explanations, as faecal matter is thought to contain entire microbial communities, which are completely transferred. However, faecal microbiomes are not synonymous with gut microbiomes, as many microbes may not make it through the digestive tract (sect. 5, para. 3). This aside, experiments transplanting whole microbiomes via faecal matter transplants or eliminating gut microbiomes using antibiotics still run into trouble when it comes to proportional causal explanations.

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To return to Yablo's pigeon, imagine you are an experimenter trying to understand what makes the pigeon peck. You present a sequence of coloured targets, and the pigeon pecks. What do you conclude? One explanation is that coloured targets make the pigeon peck. Although this accurately describes the results of the experiment, an obvious follow-up would be to try and eliminate some of the colours by presenting only component parts of the original sequence. Coloured targets may be a useful "black box" or "placeholder" explanation in this case, but any critical reader would see that follow-up experimentation is needed to identify a better causal explanation.

So are microbiome explanations simply, as Hooks et al. put it, "placeholder claim(s)" for future causal claims, typical of explanations made in a younger field? I do not believe so. In the early days of gene research, heritability estimates served as a "black box" for genetic explanations for many traits. Yet reference to "the genome" as a causal explanation did not permeate this field. Instead, causal claims about traits being primarily "genetic," later displaced with "gene for" terminology, dominated (Griffiths & Stotz 2013). This is akin to explaining brain or behavioural states as primarily "microbial" or referring to a "microbe for" certain traits.

In MGB research, a black-box strategy may be implicit for some – yet is not obvious to the majority, as community-level explanations remain rife within the literature and in the popular press. Extending Hooks et al.'s suggestions for social scientific study (sect. 8, para. 3), investigation as to why community-level explanations have dominated the field is warranted. In the meantime, researchers should strive for greater precision when communicating their research, and considering the proportionality of causal explanations will be useful for this purpose.

On the potential distortions of highly cited papers in emerging research fields: A critical appraisal

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Abstract

Citation-based metrics are increasingly used as a proxy to define representative, considerable, or significant papers. We challenge this belief by taking into account factors that may play a role in providing citations to a manuscript and whether/how those highly cited studies could shape a scientific field. A different approach to summarisation of relevant core publications within a topic is proposed.

In the target article, Hooks et al. aimed to summarise the most representative methodologies and results from the field of microbiota-gut-brain research, a controversial topic that has recently gained attention within the psychiatric community. To select the 25 most cited non-review publications, the authors performed two PubMed searches and retrieved respective citations from Google Scholar. The aim was to identify those studies that exerted a broader influence on subsequent research and that received extensive media attention. The limitation of excluding potentially relevant recent studies is acknowledged.

We recognize three major drawbacks to this literature search. First, limiting the search to only one database could be detrimental when trying to widely depict the panorama of a scientific field. PubMed relies primarily on MEDLINE, a database, which – if used alone – has been considered inadequate (Lefebvre et al. 2011). This is because of several issues that could affect search results of randomised and non-randomised studies or systematic reviews (Bramer et al. 2016; Dickersin et al. 1994; Wieland & Dickersin 2005), the impact of which on database coverage can float considerably between different topics (Rathbone et al. 2016).

From this perspective, we argue that an extension of the search to more than one database could improve the comprehensiveness of the work, potentially resulting in additional references (e.g., Berer et al. [2011], cited by more than 600 papers according to Google Scholar). This highlights the need to take into account several databases of both references and citation metrics and explore the differences (Kulkarni et al. 2009). We acknowledge that this methodology can be very time consuming, so that an optimal balance with available resources should usually be reached.

Second, descriptive analysis of top-cited articles allows for inferential thoughts on the potential relationship between style and content of a manuscript, as well as the probability of achieving citations over the years (Hafeez et al. 2019). Citation trends are potentially exposed to several biases, such as the "hot stuff" bias, the one-sided reference bias, and the positive results bias (Catalogue of Bias Collaboration 2017a; 2017b; 2017c; Greenberg 2009; Ioannidis 2005b). Hence, distortions within the citation network can generate unfounded authority of claims through highly biased, non-evidence-based information cascades and persist despite strong contradicting evidence by following publications of higher reliability (Greenberg 2009; Tatsioni et al. 2007).

A survey among more than 100 highly cited researchers assessing their top 10 cited papers hinted that top-cited publications may be generally perceived as evolutionary or revolutionary (Ioannidis et al. 2014). Also, strong claims might deceive readers into misinterpreting the manuscript as being an innovative and a pivotal step forward within that research field. Even though an association has yet to be shown, it appears reasonable to suggest that strong claiming leads to more media coverage and, perhaps, to more citations over time. Whether this is proof of a significant impact of the research itself over time remains open to debate (Ioannidis & Panagiotou 2011; Mackinnon et al. 2018): "Citation rates are determined by so many technical factors that it is doubtful whether pure scientific quality has any detectable effect at all" (Seglen 1998, p. 226). Several ways that cannot be depicted by bibliometric analyses or metrics indexes by which a research work could be significant have been described (Cheek et al. 2006).

Several known factors and possibly unknown ones can provide a paper a large number of citations. To account for some of them, an analysis on citation sources could yield interesting results. Distinguishing between citations from different publication types (e.g., reviews, editorials, original contributions), sources of citations (e.g., oneself, co-authors, and others), and related research fields (e.g., human studies, animal studies, and

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microbiological studies) at a study level and extending appraisal of scientific accomplishment beyond citation-based metrics (Ioannidis & Khoury 2014; Ioannidis et al. 2014) might have added significant depth to understanding the citation network of included studies. Hypothetically, this could influence the conclusions drawn by Hooks et al.

A distinction should, however, be made between the quality of a scientific study and its probability of being cited. Highly cited clinical research is not immune to being contradicted by subsequent studies, especially in the case of non-randomised studies and small-sample-size studies reporting large effects (Ioannidis 2005a; Tajika et al. 2015). Despite supporting the authors' view that highly cited manuscripts could exert an influence on relevant fields, a warning should be issued: They should not be considered as reliably representative of a scientific area's production or its scientific advances.

Finally, when focusing on the role that a study could play through its citations, omitting its indirect influence through reviews could bias the final interpretation. The authors' decision is understandable, because the attribution of the impact derived from a study through a review cannot be easily estimated. However, the number of published systematic reviews has increased dramatically over the past decade. Systematic reviews can both achieve a large number of citations and be regarded as increasingly pivotal in influencing the research community, both directly and indirectly through policy making. Hence, omitting their role in shaping both the core literature and the scientific panorama of the field could be considered an excessive limitation when drawing conclusions.

When arbitrary criteria are employed, unavoidable limitations are cast: The quest for comprehensiveness might not completely overcome them, but it reduces the cherry-picking bias. A comprehensive, systematic bibliometric review is therefore suggested to identify, describe, and assess influential methodologies within an emerging research field.

Practical guidelines for gut microbiome analysis in microbiota-gut-brain axis research

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Abstract

The microbiota-gut-brain (MGB) axis field is at an exciting stage, but the most recent developments in microbiota research still have to find their way into MGB studies. Here we outline the standards for microbiome data generation, the appropriate statistical techniques, and the covariates that should be included in MGB studies to optimize discovery and translation to clinical applications.

In their comprehensive review of microbiota-gut-brain (MGB) axis research, Hooks et al. raise concerns about the belated adoption of appropriate methods for studying microbiota composition. Recommendations exist – but only rarely find their way into MGB studies. Here, we point out current efforts of standardization and innovation that improve microbiota interpretability and reproducibility and provide guidelines for their application in MGB research.

Microbiome data generation involves multiple decisions concerning sample collection and storage conditions, nucleic acid extraction protocol, sequencing techniques, and pre-processing that are important to generate high-quality data and reproducible results (Costea et al. 2017). Suggestions to optimize and standardize microbiome profiling have been published (Costea et al. 2017; Sinha et al. 2017; Valles-Colomer et al. 2016; Vandeputte et al. 2017c), and, although no complete consensus is achieved, adhering to and following up on such guidelines and being aware of limitations when comparing studies are crucial. Data analysis techniques are also continuously updated, and microbiome data analysis is no exception. A pitfall of microbiome data, ignored or underestimated until recently, is that the data are compositional. That is to say that abundances of microbial groups are expressed as proportions (of reads mapping that group in relation to the total sequenced library). The application of naive normalization and statistics to such compositional data can lead to erroneous results (Vandeputte et al. 2017b). Compositionality-robust statistics were therefore recently introduced in microbiota research (Gloor et al. 2017) and have become the new standard in the field and implemented in the most popular pipelines for microbiome data analysis (Bolyen et al. 2018).

Still, when interpreting variation in microbiota composition determined by metagenomic approaches, it is important to keep in mind that the information about the microbial densities in the original sample is lost. Without microbial load information, proportional data do not allow us to draw any conclusions regarding directionality of changes. For example, an increase in relative abundance of a single microorganism could just result from it maintaining its initial numbers in a generally decreasing community (Fig. 1). Recent innovations such as quantitative microbiome profiling (QMP) (Vandeputte et al. 2017b) tackle this issue by coupling flow cytometry cell count determination with sequencing, allowing us to recreate absolute abundance profiles from proportional sequence data (Fig. 1). Besides reducing the number of false positives detected in disease association studies, the method also facilitates relating microbial absolute abundances to quantitative physiological parameters. Determination of microbial loads showed that cell densities vary greatly even in healthy subjects but are generally reduced in patients with inflammatory bowel disease. Hence, reduced microbial density could be part of a microbiota signature of disease.

Other variables can influence microbiota composition in an MG study besides the disease phenotype under investigation. In addition to confounders that are typically already taken into account in clinical studies (e.g., gender and age), microbiota-relevant factors should also be addressed in study design (e.g., by matching cases and controls) or by recording and factoring them in the statistical analyses. Gastrointestinal transit time, medication, diet, and inflammation markers should be highlighted (Falony et al. 2016;

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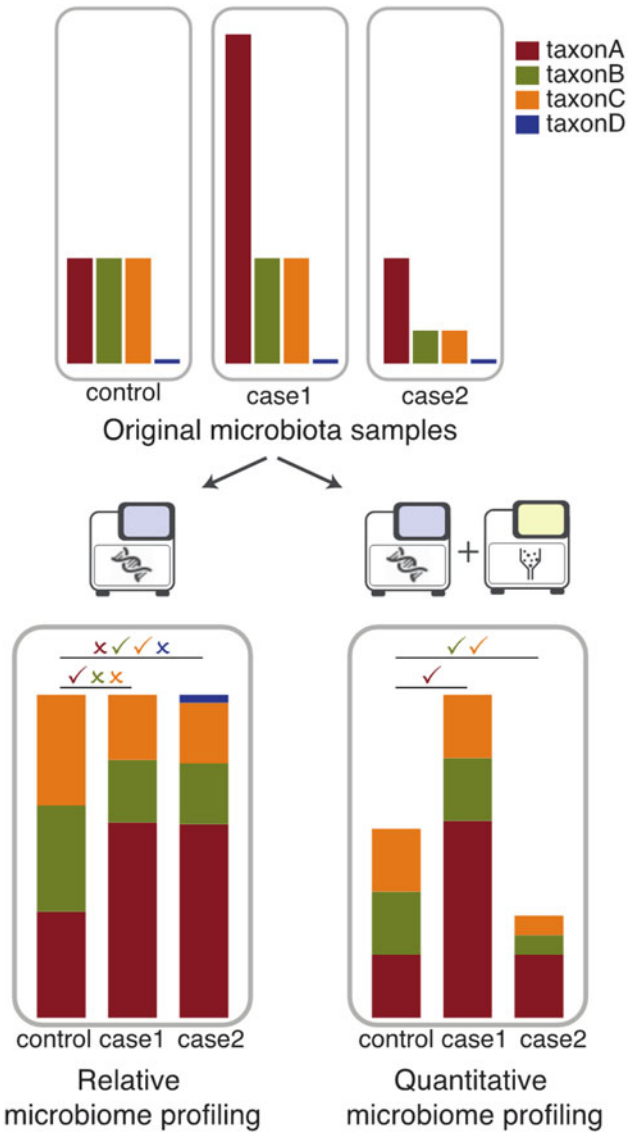


Figure 1 (Valles-Colomer et al.). Implications of the compositionality of microbiota data. Three illustrative samples (top) – one from a control (control) and two from patients (case 1 and case 2) – each containing four different microbial taxa, are analyzed by relative (bottom left) or quantitative (bottom right) microbiome profiling. In the original sample, although case 1 has an increased absolute abundance of taxon A, case 2 has decreased abundances of taxa B and C. Relative microbiome profiling results in very similar profiles for the two cases, and alongside the true differences in taxa abundances (true positives: ✓), additional apparent differences are also detected (false positives: ×). In addition, assuming even sequencing depth, samples with reduced microbial density (case 2) are more deeply sampled than the high abundance counterpart (case 1), leading to the detection of taxon D in case 2. In contrast, with quantitative microbiome profiling (coupling DNA sequencing [light blue] with cell count determination by flow cytometry [yellow]), the original absolute abundances of microbial taxa are recreated (although subsampled), and therefore, the information on directionality of the changes is recovered.

Vandeputte et al. 2015). Transit time is linked to changes in total microbial loads and in abundance of specific taxa, as the microbial ecosystem goes through different stages of development as it progresses through and remains in the intestinal tract (Falony et al. 2018). Beyond the normal variation observed in healthy individuals, altered transit time is also characteristic of several diseases, including nervous system diseases, either being accelerated (e.g., anxiety disorders; Gorard et al. 1996) or slowed down (e.g., Parkinson's disease; Knudsen et al. 2017). Therefore, to capture the disease, but not transit time-associated microbiota variations, gastrointestinal transit time needs to be tracked in MGB studies, either by measurement (magnetic tracking systems) or by using proxies such as the Bristol stool scale (Lewis & Heaton 1997) or stool moisture content (Vandeputte et al. 2017b). Additional important confounders in the MGB context include medications, several of which have been reported to affect microbiota composition, including psychotropic drugs (Cussotto et al. 2018). Effects of drugs on the microbiota can be direct, by affecting growth of specific microorganisms, or indirect, by inducing variations in transit time or host physiology (Forslund et al. 2015; Maier et al. 2018), but in any case need to be disentangled from the disease signal. Diet can also be an important confounder (David et al. 2014), especially if dietary behavioral changes are associated with the disease. Finally, inflammation has an impact on the microbiota (Cenit et al. 2017) and may not be part of the disease manifestation. Although we acknowledge the challenges of assessing dietary intake in a systematic way or controlling for it in study design, both systemic inflammation markers (e.g., C-reactive protein) and specific markers for intestinal inflammation (fecal calprotectin) measurements are straightforward.

Finally, the "causality problem" highlighted by Hooks et al. can only be tackled in study design. Strategies such as transplanting/deleting microbiota components associated with the disease to induce/reverse phenotypes in model organisms can provide valuable insights. However, it remains difficult to disentangle direct and indirect contributions of the microbiota in disease onset or pathophysiology. One way to acquire more information on potential mechanisms underlying microbiota-host associations is assessing the metabolic potential of the microbial communities under study, which requires meta-genomic shotgun sequencing. Although computationally more challenging and only rarely performed in MGB studies, such data are very valuable to study the most direct of the proposed microbiota-driven route of MGB communication: the microbial synthesis and degradation of neuroactive compounds (Lyte & Cryan 2014). Future research will become easier as context-specific tools are developed, such as the recent publication of neuroactive compound metabolism of the human gut microbiota (Valles-Colomer et al. 2019), which provides a catalog to facilitate future MGB shotgun meta-genomic analyses.

The MGB field is at an exciting and promising stage. An early adoption of the latest advances in microbiome research by the MGB community, with careful study design, appropriate analysis techniques, and taking into consideration known potential confounders, will promote reliable discovery and lead to earlier translation to clinical application.

Inter-individual variation shapes the human microbiome

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Abstract

The target article suggests inter-individual variability is a weakness of microbiota-gut-brain (MGB) research, but we discuss why it is actually a strength. We comment on how accounting for individual differences can help researchers systematically understand the observed variance in microbiota composition, interpret null findings, and potentially improve the efficacy of therapeutic treatments in future clinical microbiome research.

Individual differences, such as diet, biological sex, and social behaviors, moderate the effect of the microbiome on psychological and biological variables. Although the target article occasionally alludes to the importance of considering the effect of inter-individual variability, it does not provide a particularly useful or nuanced discussion of how accounting for this variability can improve the predictive power of microbiome-related variables on clinical, biological, and psychological outcomes, as well as help make sense of null effects currently reported in the microbiota-gut-brain (MGB) literature. In this commentary, we will briefly review how exploring inter-individual variability provides an opportunity for scientists to probe more deeply into the relationship between hosts and their microbes.

Microbiologists are already exploring the effects of individual differences on the microbiome. In a paper the target article referenced, Clarke et al. (2013) demonstrated that biological sex moderated the relationship between bacterial presence (germ free vs. conventional) and neurometabolite levels in mice exposed to stressors. In another study, Benton et al. (2007) found that overall, probiotic treatment did not have a significant effect on psychometric measures. However, when they accounted for baseline mood, they discovered the treatment did improve the mood of people whose mood was especially poor at baseline. By accounting for individual differences, these authors were able to extract meaning from what otherwise might have been null effects.

In two recent publications, researchers found further evidence that accounting for inter-individual variation is key to uncovering important relationships in MBG research. When Dill-McFarland et al. (2019) examined the microbiome of romantic couples, they found that couples had more similar microbiomes *only* when they reported more relationship closeness. There were no differences in similarity between couples reporting somewhat close relationships and unrelated individuals. Jadhav et al. (2018) discovered that striatal dopamine receptors were correlated with microbiome composition, but only for the 15% of rats that exhibited compulsive, as compared to typical, alcohol consumption behavior.

When examining inter-individual variation in healthy populations, Falony et al. (2016) found 69 clinical and questionnaire-based covariates were associated with microbial composition at a 92% replication rate. These covariates ranged from biological factors, like stool consistency or medication use, to lifestyle factors, like having pets or one's chocolate preference. Falony and colleagues argue that these covariates must be accounted for when examining the microbiome of individuals with medical issues, as they can explain a significant portion of the variance observed in the microbiome independent of disease presence. This argument is not unique, as scientists have also pushed for exploring how inter-individual variability in biological and lifestyle factors interact to influence MGB-related outcomes (Wissel & Smith 2019, p. 13).

As the target article points out, the media can often sensationalize MGB findings, especially for the therapeutic potential of targeted microbiome treatments. Accounting for inter-individual variability may help transform these sensationalized promises of MGB therapeutic treatments into viable therapeutic practices grounded in careful science. Perhaps the most interesting context in which to study this is the case of fecal matter transplants, or FMTs, in which the microbiome is transferred from one person to another. FMTs are used to treat severe gastrointestinal (GI) disease, such as *Clostridium difficile* infection, when conventional treatments, such as antibiotics, fail. There are very strict guidelines that donors must meet for their stool to qualify for transplantation. However, none of the exclusion criteria include individual difference measures of mental health. In fact, inter-individual variability in mental health is almost never a factor in donor qualifications, which is quite surprising because there are clear and consistent findings that mental health is associated with microbiome composition (Liu 2017). Because these important individual differences are not measured at all in most FMT cases, researchers would have no way of gauging which donor traits are transferred along with the FMT or if it is even possible to effectively shift recipient traits with the procedure.

This has two major implications. The first is the potential harm physicians may be causing their patients by not collecting these individual difference measures. For example, a physically healthy person can have non-clinical (or even clinical) levels of anxiety, which is often associated with specific microbial profiles. It has been shown in mice that an FMT is sufficient for transferring these anxious traits (Bercik et al. 2011b), so one would think physicians would want to know if this same transfer is possible in humans, and if so, prevent it. The second implication is the potential for MGB to treat illnesses outside of GI disorders. One of the top and most discussed contenders is treatment-resistant depression. If it *is* possible to improve psychiatric symptoms with FMTs, this could potentially revolutionize the approach physicians take to treat illnesses resistant to conventional therapies. The FMTs actively being conducted provide the perfect opportunity for collaboration between physicians to measure the microbiome and psychologists to account for mental-health-related individual differences.

MGB research is in a period of rapid growth, and findings can become outdated before they are even in print. The target article focuses on 25 papers that were groundbreaking when published but, as science has progressed, have become outdated themselves. As such, the target article misses many of the nuances related to inter-individual variability currently developing in the field. By systematically accounting for meaningful individual differences, researchers can begin to better understand the humans behind the microbes.

Authors' Response

Causal clarity and deeper dimensions in microbiota-gut-brain research

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Abstract

Our analysis of microbiota-gut-brain (MGB) research took MGB to task for some of its methods, concepts, and interpretations. Commentators then raised numerous issues about the neuroscientific and microbiome aspects of MGB and how it can be understood as a field. We respond by addressing the dimensionality (scope and depth) and causal focus of MGB.

R1. Introduction

Human microbiome research has captured the imagination of scientists, clinicians, research funders, health providers, and the public. There are many good reasons for such enthusiastic responses to the early insights generated from closer scrutiny of our microbial residents. Not only has a great deal been learned about these multitudinous occupants of human bodies, but there are also anticipations of new explanations and treatments for a vast range of diseases and disorders. Although little of this promise has been precisely actualized yet, extensive efforts are underway to bring microbiomes into deeper and sharper focus. This "second phase" of research in the emerging microbiome arena is now occurring in one of its newer sub-fields: microbiota-gut-brain (MGB) research.

The broad scope and interdisciplinary appeal of MGB research are evident in the commentaries on our target article. Our aim in that paper was to raise issues of importance for anyone interested in this area of scientific research, with the expectation that further cross-disciplinary dialogue will contribute to problem solving and future advances. The commentaries bear out this aim: Each of them constructively suggests ways in which MGB research can avoid existing pitfalls and explore new areas of investigation.

We see three main themes in these commentaries, with several papers contributing to more than one of them (Table 1). Theme 1 is "expanding the neuroscience of MGB research," particularly to investigate relationships between microbiomes and brain development and increase insight into potential treatments. Theme 2 is concerned with "expanding the microbiome analyses of MGB research" by developing additional bioinformatic, experimental, and explanatory tools. Theme 3 focuses on "understanding MGB as a field" from bibliometric, translational, and terminological angles.

Theme 1: Expanding the neuroscience of MGB research

One of the strongest areas of advocacy in the commentaries is for closer attention to brain development in MGB research. **Johnstone & Cohen Kadosh**, for example, propose that developmental cognitive neuroscience can make MGB research sounder and stronger. **Howell & Tramacere** also call for more attention to behavioural development in relation to MGB research, especially with regard to longitudinal investigations in human subjects. They believe that microbiomes and brain development are linked to breast milk and are very enthusiastic about recent findings in Carlson et al. (2018), as are **Kelsey & Grossmann**. Kelsey & Grossmann go on to urge "mapping [of] the development of the microbiota-gut-brain axis during human infancy." They criticize us for having "overlooked" the role of the human microbiome in brain and cognitive development, although they are blaming the messenger here. As **Alberts, Harshaw, Demas, Wellman, & Morrow** (**Alberts et al.**) observe, it is the 25 most

Table R1. Three general themes categorizing the commentaries on the target article "Microbiota-Gut-Brain Research: A Critical Analysis" by Hooks et al.

Theme 1: Neuroscience+	Theme 2: Microbiome+	Theme 3: MGB as a Field
Neuro/cognitive development Johnstone & Cohen Kadosh Azhari, Azizan, & Esposito Kelsey & Grossmann Howell & Tramacere Alberts, Harshaw, Demas, Wellman, & Morrow	**Microbiome analyses** Alberts et al. Wissel & Smith Valles-Colomer, Falony, Vieira-Silva, & Raes Blakeley-Ruiz, McClintock, Lydic, Baghdoyan, Choo, & Hettich	**Bibliometric issues** Borghi et al. Ostinelli et al. Wissel & Smith Alberts et al.
Techniques and mouse models Aarts & El Aidy Alberts et al.	**Microbiome function** Andreoletti & Rescigno Blakeley-Ruiz et al. Aarts & El Aidy	**Translation: from multidisciplinarity to communication** Andreoletti & Rescigno Gligorov Birk Wissel & Smith
Neurotherapies: probiotics and prebiotics Borghi, Vignoli, & D'Agostino Korte & Korte Dalile, Van Oudenhove, Verbeke, & Vervliet	**Microbiome causality: specific entities** Fetissov Clark Lynch Dalile et al. **Causal explanation and complexity** Johnstone & Cohen Kadosh Lynch Borghi et al. Valles-Colomer et al.	**Terminology: stress** Birk **Dysbiosis** Johnstone & Cohen Kadosh Andreoletti & Rescigno

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cited MGB articles that have given cognitive development little attention. Our target article did in fact address some conflicting claims in those 25 papers about critical neurodevelopmental periods.

Azhari, Azizan, & Esposito (**Azhari et al.**) start out somewhat differently, by saying that our target article proposes that "the mere presence" (para. 1) of gut microbiota should be considered developmental signals and not specific causal agents (although we do not say exactly this, we are happy to listen to evidence and reasons for this position). Azhari et al. go on to argue that although the fetus does not have a gut microbiota (despite some artefactual findings), maternal inflammatory responses to bacterial or viral infection need prime consideration with regard to their impact on prenatal neurodevelopment. Azhari et al. are thus urging us (and MGB researchers) to consider the microbiota as an "intermediary system," rather than a "direct causative agent" in the case of neurodevelopmental disorders such as autism.

Fetal responses to maternal inflammation may indeed be one avenue of insight into cognitive neurodevelopment. Earlier fields, such as psychoneuroimmunology, successfully showed how maternal infection-induced inflammation alters offspring behaviour. However, many MGB researchers might worry that **Azhari et al.**'s focus on maternal inflammatory products puts the microbiota at a causal remove. In other words, the microbiota, or specific components of it, become not the most proximate causes of particular phenotypes but more distal causal contributors. Not everyone will see this as the right way to understand microbiome causality, which is a topic we discuss at length in Theme 2. Moreover, we are not as sure as Azhari et al. that "specific species of microbes" are not key parts of these causal chains. Although an increasing amount of MGB research refers to neuroimmune interactions, a pressing question is whether and how the experimental investigation of inflammation can be integrated with broader microbiome surveys. As we discuss in Theme 2, homing in on testable causal factors is not so easy when the microbiota is seen as an aggregated mass of potential causality that is embedded in diverse systems of causal interaction.

Assessing causal contributions from microbiomes is crucial whatever the phenomena being explained. MGB and other microbiome research is concerned not only with disease, but also with "normal" human features. An impressive number of our commentators are keen that our reflections on MGB research include additional attention to the relationship of microbiota to cognitive development (see Table 1). We are happy to oblige by focusing on a paper acclaimed by two commentaries as exemplary: Carlson et al. (2018). This paper examines the microbiomes of 1-year-olds. It finds there are different "types" of microbiome composition (similar to adults), and that breastfed children have lower-diversity microbiomes that also correlate with greater language development at 2 years old. Although this study certainly has some promising elements, we are not sure it will push MGB onto a better track in the ways **Howell & Tramacere** and **Kelsey & Grossmann** believe it will. Let us outline briefly why.

First off, Carlson et al. (2018) are focused on correlations, in this case primarily between microbiome diversity and cognitive markers (language). The study explicitly denies positing a causal relationship but uses other terminology to hint at such connections (e.g., "the development of the gut microbiome … support [s] cognitive development" [p. 157]; "gut microbiota influences brain development during a critical period" [p. 154]). But, in fact, all these correlations could also be explained by other variables measured in the study, particularly the nutritional benefits of breast milk and – more implicitly – parental care regimes (see our Fig. 1). It is not clear why microbiota were not sampled at 2 years (when language development was assessed), especially when the authors push hard on a stage-like model of microbiome development (e.g., "delayed maturation of the microbiome" [p. 154]).

We agree that it is interesting to find less diverse microbiomes correlating with the desirable host state (more language), but this is simply because it inverts the often fallacious assumption that less diverse microbiomes correlate with disease (see, e.g., **Johnstone & Cohen Kadosh**; for why this is a fallacy, see Shade 2017). Making lower diversity "positive" probably does not avoid the shallowness of diversity correlations with host states. A potential treatment suggested by Carlson et al.'s findings is that the repeated administration of antibiotic treatments could deplete the infant's microbiota and raise cognitive attainments. The authors do not state this explicitly, but they discuss how antibiotics "delay microbiota maturation," that "immature" microbiota are less diverse, and that less diverse microbiota associate with higher cognitive outcomes. The final inference is easy to make. Fortunately, nobody is yet proposing this therapy, which indicates to us that the study's findings are recognized as missing crucial variables and pathways. In short, although we feel sure that attention to cognitive development is a good aspect to elaborate further in the MGB literature, study designs of this sort are more likely to add to the litany of problems outlined in our target article rather than solve them.

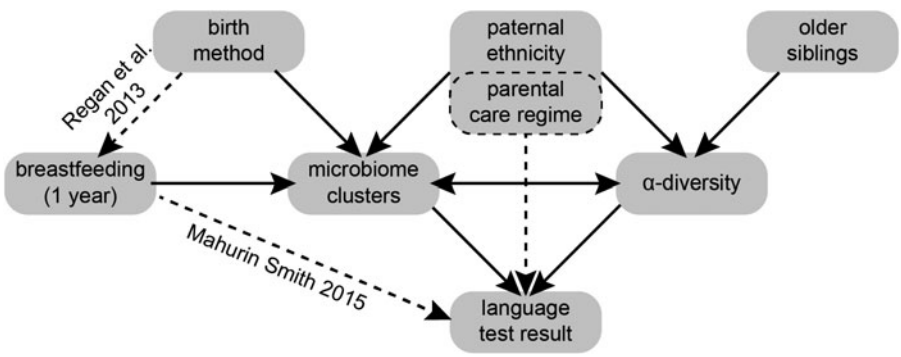


Figure R1. Correlations revealed in Carlson et al. (2018) are represented by *solid lines*, and those previously reported in the literature are represented by *dashed lines* (e.g., Mahurin Smith 2015; Regan et al. 2013). *Arrows* indicate the most likely directions of influence (positive and negative). The *double arrow* between "microbiome clusters" and "α-diversity" indicates that although the paper finds these measures to be "independent," the relationship between the two is necessarily mutual. Carlson et al. mention parental care regimes only implicitly (i.e., "environmental factors associated with this sociocultural construct" of ethnicity [p. 154]). We take this to mean that different ethnic backgrounds can impact key variables such as how parents support infant learning and how they follow nutritional and medical guidelines.

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R2.1. Techniques and mouse models

Other commentaries focus more closely on specific neuroscientific techniques that could potentially contribute to MGB insights. **Aarts & El Aidy** suggest that brain imaging in the form of functional magnetic resonance imaging (fMRI) may increase the depth of neuroscientific knowledge in microbiome-related investigations. They cite a handful of studies that have done just that. Even though there are limitations to what fMRIs reveal (Klein 2010; Logothetis 2008), we agree that brain-imaging methods have the potential to provide more detail about the activation of brain structures in relationship to microbiome conditions. Other visualization techniques (not just fMRIs) might also reveal associations between visualized brain states and microbiome composition; again, they may not (e.g., Carlson et al. 2018). Although everyone has his or her favourite technique, it is not always the case when a field is new and still developing that "more is better" all the time. Moreover, it is not at all clear that additional types of data are what are needed to push microbiome-brain findings over the causal threshold.

Alberts et al. emphasize the developmental importance of parent-offspring interactions for gut microbiome colonization and make a plea for more careful behavioural approaches. They are disappointed that we believe there are limits to mouse models of human disorders, which they think is not justified because of the "evolutionary conservation of core mechanisms" (para. 7). The problem we see is that there is little evidence that these claims are true for many aspects of behaviour. Assuming shared evolved mechanisms for human and rodent behaviour is part of the methodological and translational problem indicated by our target article. Alberts et al. go on to argue that "sophisticated experimental" use of germ-free animals will advance the field and allow mechanisms to be postulated and therapies to be discovered. They suggest the answers lie in "methods that are sensitive, objective, and sufficiently nuanced to capture social behaviour" (para. 3).

In contrast, we believe that even optimal methods for measuring social behaviour in mice are just as likely to provide evidence for *species-specific* behavioural patterns, rather than conserved core behaviours relevant to all mammals. These species-specific behaviours will probably be much harder to compare to human behaviour in general, let alone mental disorders, than the study of one or two parameters in rodents that are supposed to reflect one symptom of a human condition. In addressing this issue of human-mice comparability, **Kelsey & Grossmann** argue that we should have paid more attention to "humanized mouse models" (para. 4), in which human microbiota are transferred into mice. Possibly, but there are also well-known problems in transplanting human microbiota into mice (see Arrieta et al. 2016; Nguyen et al. 2015). These problems greatly exacerbate the basic translational obstacles we noted in our target article; **Birk** also points to these challenges. Mouse-human translation becomes even more troublesome when treatments derived from mice studies are applied to humans. A recent study (also noted by **Korte & Korte**) demonstrates how probiotics often fail to colonize mice intestines, whereas humans may have far more individualized colonization responses and hence different modes of potential host impact (Zmora et al. 2018).

R2.2. Therapies

The greatest treatment hopes of MGB and some broader microbiome research are probiotics and dietary interventions. **Kelsey & Grossmann** think the probiotic aspect of MGB research is "somewhat overemphasized" (para. 5). We concur, on the grounds that these are not straightforward therapies, as our target article discussed. **Korte & Korte** agree and go on to urge more careful hypotheses about how probiotics might affect host physiology. They encourage attention to new research that reveals a "personalized" response to probiotic therapy (Zmora et al. 2018). Therapeutically, this means that different strains of probiotic bacteria (i.e., below the species level) may have to be used on very specific populations of people. A personalized approach also means that different probiotic organisms may help different conditions, and that detailed diagnosis and treatment plans would have to accompany any therapy. Doing so would undermine expectations of generic probiotics able to provide a fast universal fix of conditions ranging from anxiety and Alzheimer's to schizophrenia and Tourette's syndrome. The scientific detail required for personalized probiotics undermines the DIY appeal of such therapies. More personalized probiotic treatments might also not be so attractive to commercialization (see the Supplementary Material that accompanies our target article) because of decreased consumer bases.

Expanding the treatment theme further, **Dalile, Van Oudenhove, Verbeke, & Vervliet** (**Dalile et al.**) note that our target article ignored research on prebiotics, which are foods believed to favour "good" microbes in the gut; prebiotics may also refer to any dietary fibres that are primarily broken down by microorganisms. It is true we do not even mention prebiotics in our target article, but that is simply because the 25 most cited MGB articles paid them no attention. Dalile et al. argue that prebiotics are therapeutically better than probiotics, and to substantiate their claim, they discuss a systematic review of prebiotic-microbiome studies. Although it is always useful to draw on comprehensive analyses of a topic, we are concerned that many of the problems present in probiotic research would manifest themselves in prebiotic and other food-based studies. Dalile et al. suggest that prebiotics are good therapy because they increase the numbers of certain bacteria (or their fermentation products) that correlate with neurobehavioral benefits. However, it is not obvious to us how universally "good" any strain of bacteria can be for mental health; plus, dietary confounding is a recognized problem in microbiome research. In a nutshell, we do not think there is sufficient evidence of sufficient quality to warrant Dalile et al.'s conclusion that "prebiotics have a higher potential than probiotics [for] causal claims" (para. 6) about microbiomes, brains, and behaviours.

To add to the complications of microbiome-oriented therapies, **Borghi, Vignoli, & D'Agostino** (**Borghi et al.**) note that poor nutritional and feeding habits are common in patients with mental illness. Their commentary then provides more data showing correlations between diet and mental health disorders. We think they may be implying that altered diet can be a common cause that leads both to host disorders *and* "reduced diversity microbiota." This is by no means the same as saying the microbiota is the causal agent; instead, it could be merely a causally unrelated correlate of disease. We address the issue of causality in more detail in Theme 2 but agree that however microbiome causation of disease is understood, it must be investigated in the context of diet. Most human microbiome research does this already but does not always carefully analyse the causal structure of the relationships between diet, microbiome composition, and health (Lynch et al. 2019). Other commentators (**Valles-Colomer, Falony, Vieira-Silva, & Raes** [**Valles-Colomer et al.**]; **Aarts & El Aidy**) also point to additional confounders in microbiome

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studies such as intestinal transit time, inflammation, and medication. More attention to causal structure (i.e., common causes, confounders, causal order, and causal interactions) would certainly help with the evaluation of causal hypotheses in MGB and other microbiome research.

R3. Theme 2: Expanding the microbiome analyses of MGB research

We are fully aware that microbiome research is by no means standing still and it is good to hear more about improvements. Several commentaries elaborated on how methods of microbiome analysis have advanced, even in the last year or two. **Valles-Colomer et al.** outline evolving methods and standards for microbiome data generation and analysis, and so do **Blakeley-Ruiz, McClintock, Lydic, Baghdoyan, Choo, & Hettich** (**Blakeley-Ruiz et al.**). Echoing them, **Alberts et al.** suggest that Knight et al. (2018) point the way toward better microbiome methods. We also cited this paper in our target article as one of several recent reviews that discuss progress in the context of problems that remain to be solved.

A major methodological issue has to do with the quantification of microbiota and microbiomes. Both **Borghi et al.** and **Valles-Colomer et al.** mention that the density of intestinal microbiota ("microbial load") varies substantially among healthy people and is consistently lower when hosts have disorders such as anorexia nervosa or inflammatory bowel disease. Standard sequencing analyses do not take such quantitative characteristics into account. More generally, because microbiome sequence data are usually expressed as ratios, even the overgrowth of one bacterial species can bias reported proportions of taxa and thus increase false discovery rates of patterns associated with diseases. Although there are now statistical tools that can deal with such issues (see Knight et al. 2018), Valles-Colomer et al. discuss the advantages of absolute quantifications of bacterial taxa. These quantifications can be achieved by supplementing sequence data with direct cell counts. Not only does this method overcome proportionality biases, but it also adds information on the quantity of bacteria present, which may indeed be relevant to disease outcomes in the host.

Wissel & Smith focus on a different problem of relative differences in microbiota. They claim that our "article suggests inter-individual variability is a weakness of microbiota-gut-brain (MGB) research" (abstract). To clarify this point, we believe person-to-person variation in microbiome composition is an inevitability that is difficult to deal with and is seldom dealt with adequately. Wissel & Smith report that some MGB research does indeed engage with the inter-individual variation of microbiome composition, but all their examples are class based rather than individual based (e.g., fairly coarse microbiota differences classified by host sex or host "mood"). We think these commentators are using the term "inter-individual variability" in a different sense than is usually the case in microbiome research. It is an important topic: on that we agree.

What is standardly meant by inter-individual variability is that each person possesses a unique and fairly stable gut microbiome. The taxonomic composition of microbiomes in the intestines of any two human hosts may look quite different from each another, which means it is difficult if not impossible to find clear microbiota "types" that are reliably associated with general host states (Gilbert et al. 2018; Human Microbiome Project Consortium 2012). Implying there is a generally "optimal" microbiome for a particular phase of life (as **Howell & Tramacere** suggest) is quite problematic in light of this variability. At present, there is no real consensus on how to deal with the inter-individual variability of the microbiota. Taking a very coarse-grained taxonomic approach in order to find more similarities between individuals leads to coarse-grained findings, some of which are cited by **Wissel & Smith**. The most successful approaches so far have focused on specific bacterial taxa within each microbiota, or they have attempted to analyse bacterial function.

Function refers to the ways in which microbiota can interact with and affect the physiology of the host, immunologically and metabolically. **Andreoletti & Rescigno** believe that a taxonomy-based approach "has been abandoned already in favor of a functional account" (para. 5). However, it is well-known that a large amount of microbiome research continues to use biomarkers for taxa, as, for example, **Valles-Colomer et al.** observe. Even when microbiome analyses are concerned with function, the situation is not straightforward. Currently, the functions of sequenced genes in the microbiome are predicted by comparing those sequences to similar ones that have a known function (i.e., from earlier experimental evidence or bioinformatic predictions). However, many microbial genes have not been studied in sufficient detail and so are designated "unknown" and excluded from the analysis. In other words, much functional information is still missing.

Recently, researchers have begun to investigate not only the simple presence of genes in microbiomes, but also the completeness of the functional pathways in which they act (Franzosa et al. 2018). Doing so changes the focus from reporting the presence of one or two genes in the same pathway to evaluating whether the right genes in sufficient numbers are present in the microbiome for various multistep functions to be actualized. However, this approach raises yet another issue of treating the microbiome as a "soup," in which each microorganism, gene, or metabolite is free to interact with all the other components. This is not the case in the mammalian gut, which is compartmentalized by the epithelial gut barrier and different layers of mucus.

Most importantly, the mere presence of a gene in a microbiome is no indication that it is functional within the gut. A better focus is gene expression, which if it occurs suggests function, especially when expression profiles are coupled with presence of either proteins or metabolites that are linked to gene action. **Blakeley-Ruiz et al.** advocate more attention to the "active" aspects of the microbiome as divulged by methods called metatranscriptomics (analysis of all the transcription products of microbiome genes), metaproteomics (analysis of all the expressed proteins of microbiome genes), and metametabolomics (analysis of all the metabolic products of microbiome reactions). These newer techniques are indeed promising, but each of them in turn has inherent issues to do with data collection and interpretation (Knight et al. 2018). Most problematically, these data-intensive methods increase the scale and complexity of data gathering and analysis, which is already a challenge just for gene sequences (see **Aarts & El Aidy** for a discussion).

Much microbiome research outside MGB can be thought of as "data driven" rather than experimentally driven. Large-scale molecular studies (such as metatranscriptomics, etc.) can be complementary to hypothesis-driven research, meaning the two can work in tandem: The former can describe the state of the system and generate possible hypotheses for further examination, whereas the latter can answer very precise questions about that system (Kell & Oliver 2004). But large data-driven studies are

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sometimes dressed up in the literature as hypothesis driven when in fact they are merely fishing around for hypotheses. **Aarts & El Aidy** mention how hypotheses can be formulated post hoc and then presented as if they drove the study in the first place. In addition, say Aarts & El Aidy, only some significant results from a variety of statistical tests may be presented and the non-significant findings not even mentioned. "Hypothesis-driven intervention" is **Blakeley-Ruiz et al.**'s more general suggestion for the future of microbiome research (para. 8), but this oft-preferred view of scientific practice is not so easy to implement when large-scale data sets and hundreds of variables are involved, as **Borghi et al.** point out.

R3.1. Causal explanation

As we emphasized in our target article, a major issue in all microbiome research, and particularly MGB research, is how to detect and evaluate causal relationships. When seeking ways forward, it can be useful to turn to philosophy of science and see what it has to say. **Korte & Korte** suggest that "the field of MGB research would greatly improve when more attempts are made to falsify the hypothesis (Popper 1963)" (para. 1). A flood of philosophical ink has been spilled over the last few decades on examining Popper's rather appealing characterization of good science. The general conclusion of this massive literature is that Popper's abstract methodology of falsification is an ideal of good science that simply does not really reflect how science works; even Popper eventually reached this conclusion (Thornton 2018). This is especially the case in today's life sciences, which value positive findings and may not always be driven by hypotheses at every stage of inquiry. Nevertheless, without worrying too much about the truth or falsity of "falsificationism," it is good general practice to try to formulate and test hypotheses about causal processes. For us, the question is whether broad reference to "microbiomes" (compositional or functional) will really allow causal hypotheses to be tested and clear conclusions drawn.

This key topic emerges in a subset of commentaries that examine whether microbiomes as a whole are the causal agents of brain-behavioural disease and health states, or whether specific components of the microbiome are what should be understood causally. In tackling this topic, **Lynch** addresses whether whole microbiome explanations will succeed, or whether explanations involving particular microbes (or small groups of different microbes) are more likely to be successful in accounting for host phenomena. Using the criterion of "proportionality" (found in explanations in which causes are proportionate to the effect), she suggests smaller-scale causality is the way forward for MGB. However, Lynch also notes how microbiome research (or its media uptake) is particularly attached to much broader and vaguer "microbiome" explanations. This is rather odd in light of a historical view of genomics, she observes, which has always sought proportionate gene-based explanations rather than disproportionate whole-genome ones. Some sociology of science may eventually illuminate this curious trend in microbiome research.

Clark largely echoes **Lynch**'s advice, pointing specifically to neurotropic enterovirus research as "demonstrating causal relationships between gut-brain-axis status and infected host health, development, behavior, and mind" (para. 2). Microbiome researchers used once to focus exclusively on the bacterial components of gut microbiota, but these days, they are increasingly concerned with the viral components (or "virome") of gut microbial communities. A "virome" focus seeks to understand not only all of the viral groups in the gut, but also their interactions with prokaryotic and eukaryotic microbes in relation to host health (Pfeiffer & Virgin 2016). These interactions are barely understood at present, largely because experimental exploration of the virome is limited. Although work on neurotropic enteroviruses precedes microbiome research by many years and is limited to a very specific range of viruses, it is an example of how insights into causal relationships are facilitated by having a more specific experimental focus, be that organismal, viral, or molecular.

Fetissov too argues along these lines when he proposes that microbiota effects on behaviour are potentially related to the generation of antibodies against gut microbial components, which then recognize mammalian peptide signalling molecules. Although any particular claims about causal entities and mechanistic pathways might be questioned, homing in on specific relationships such as a recognized group of organisms, molecules, or viruses is likely to be more experimentally tractable and generate more precise findings. Conversely, after proposing neurotropic viruses as causal agents of disease, **Clark** dwells on "cosmopolitan virus-microbe relations" (para. 2), which are again broad and unclear. We suggest that as soon as the research focus moves to this more general and unspecified level, problems with attributing causality are likely. **Alberts et al.** think researchers can have it both ways: They can consider "the microbial community in its entirety" even while looking for "missing or over-represented" microorganisms that just might be implicated in host phenotypes (para. 5). This is reasonable advice, but being able to do the latter already relies on decomposing the "entire community" and knowing what each individual microbe does to the host. MGB and other microbiome research is not able to do this yet except for a small selection of microorganisms.

In a similar vein, **Johnstone & Cohen Kadosh** believe that developmental cognitive neuroscience (DCN) is well equipped for "establishing causal relationships between dysbiosis and mental health problems" (para. 5). They see DCN as multilevel and multifactorial in its explanations, and argue that this allows the specification of mechanisms and the identification of causal relationships from correlational ones. Although strategies to improve the scientific understanding of complex phenomena are essential, DCN is probably not alone in having the potential to provide a helpful perspective. Psychoneuroimmunology, for example, is another field that may be operating in a similar spirit (e.g., Konsman et al. 2002). Although Johnstone & Cohen Kadosh's short commentary is not detailed enough to lay out how such causal explanations have been achieved in DCN, we doubt that any of its putative explanatory success depends on positing large-scale complex entities without decomposing them and experimenting with individual components one by one. After that, of course, these components can be *recomposed* into larger networks of causal influence, but the initial breakdown is crucial for detecting experimental effects. We fully acknowledge **Borghi et al.**'s emphasis on the complexity of interactions between the gut, brain, and microbiota but think that a more traditional emphasis on the experimental targeting of proportionate and specific causes will make steps in the right direction.

R4. Theme 3: Understanding MGB as a field

As we noted several times in our target article, MGB is still an emerging field of research. That means snapshots taken now of its achievements will not necessarily match what it becomes

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tomorrow – in particular, it means that current failings do not necessarily dictate the future of MGB research. Our commentators address several aspects of how a new field such as MGB can be understood and elaborate on the problems that plague this formation process.

R4.1. Bibliometric issues

Ostinelli, Gambini, & D'Agostino (**Ostinelli et al.**) find that our bibliometric methods are deficient, particularly because at least one highly cited MGB paper was missed in our original analysis. To them, this indicates "cherry-picking." Moreover, they argue, high citations are not good sources of evidence because the former can occur for bad reasons (provocation, etc.). They believe we did not distinguish adequately between article types or citation sources. They also suggest we should have included "systematic reviews" and not excluded the large numbers of MGB reviews our literature analysis found.

Borghi et al. also say that a focus on top-cited papers is bad methodology. Moreover, recent MGB literature has better microbiome analyses. They say generally that MGB is a new field and that it is just a matter of time before the research situation improves. **Wissel & Smith** echo this position when they say that looking at top-cited papers is biased in favour of outdated ones. **Clark** also notes that our perspective is based on "25 narrowly chosen literature examples" (para. 2), and he prefers to pay attention to some less cited material that is able to demonstrate causal relationships (see Theme 2).

These comments require us to reiterate the "snapshot" methodology of our target article. As we stated in our target article, we selected these 25 papers because of their centrality to the established experimental core of MGB. Their "most cited" status indicates these papers have influenced the field, in terms of their findings, methodologies, and conceptual machinery. By focusing on them, we hoped to delve more deeply into an influential corpus of work than we could have if we had conducted only a shallow survey of the entire body of literature. It did not escape our attention that a focus on "top-cited" papers would mean leaving aside more recent and potentially improved work in the field. We do not believe that citations are the apotheosis of bibliometric analysis, but citations do mean researchers are paying attention and often gaining inspiration and structure from these influential papers.

We also made very clear that we were not carrying out a systematic review, and even that we thought it was probably too early to do this for MGB, which is not only fairly youthful in its career, but also quite diverse. It will be difficult to compare effectively its different methods, data, and hypotheses not only now, but also for the field as a whole in the future. However, we take this opportunity to remind readers of systematic reviews and meta-analyses of microbiome and obesity correlations and experiments, which showed that accumulated findings were inconsistent, whether concerned with bioinformatic patterns or experimental effects (Duvallet et al. 2017; Sze & Schloss 2016). MGB research is concerned with many more host phenomena than obesity, but we suspect that sub-categories of research (e.g., depression-related MGB) might find themselves in the same position as obesity when systematic reviews and meta-analyses are eventually conducted.

We further note that the highly cited paper **Ostinelli et al.** say our methodology missed (Berer et al. 2011) did not fit our specifications. We searched for highly cited articles linking microbiota/microbiomes and brain-behaviour phenomena. The "missed" article is about connections between the microbiota and multiple sclerosis, which is a neurological disorder rather than a behavioural or mental state. From our perspective, it is therefore not fully relevant to the body of work we loosely refer to as MGB. In other words, although we do not claim that our bibliometric exploration of the field is perfect, this single example of an important but omitted article is not compelling. However, perhaps this means that Ostinelli et al. have a different conception of what counts as MGB and think it should include basic neurological disorders. We agree we did not make this exclusion as clear as it should have been in the target article.

Clark makes a general point about whether top-cited articles can accurately depict trends in a field. His solution is to turn to meta-analyses, particularly to capture less cited but more robust findings. As we said in our target article and again in this response, there are major issues of when it is timely in the emergence of a field to do such studies. One consequence of thinking it is too early for systematic reviews and meta-analyses might be that MGB research is not yet a fully formed field. There are no universal definitions of what constitutes fields of research, but it is probably unobjectionable to describe them as loose collective research endeavours that use different tools to illuminate shared but fairly general research questions. Most clearly, perhaps, a research field tends not to be described as an established discipline and hence is free to draw on many different disciplinary tools and perspectives. This proclivity is both a strength and (sometimes) a weakness of MGB research.

R4.2. Translation

Translational issues abound in MGB research, where they range from mouse-human translation problems to how scientific findings are translated across disciplines and then into media reports, everyday talk, and health regimes (all discussed in our target article). Issues of multidisciplinarity lie on the "inward-facing" end of translation, which is concerned with overcoming internal barriers to scientific success. Put another way, translation needs to occur between disciplines for MGB to work. **Andreoletti & Rescigno** raise the possibility that the issues discussed in our target article are "typical of an *emerging multidisciplinary field*" (para. 1). They recommend seeking advice from experts to improve matters. To us, this recommendation falls a bit short with regard to the practical demands and everyday difficulties of multidisciplinary research. **Aarts & El Aidy** have narrower but more concrete suggestions that revolve around trial registration and preregistration. They believe that having formal requirements for clear hypotheses in advance of inquiry will help the field develop more rigorously as disciplines continue to interact and make potential MGB findings.

On the outward-facing (or publicly oriented) end of the translation spectrum, **Gligorov** speculates as to whether or not commonsense conceptions of health are revised by scientific discoveries. She distinguishes between easily revised common sense (beliefs that share similarities with scientific findings) and more intransigent common sense (beliefs that are in opposition to scientific findings). We are sure it will be worth doing some solid research on this topic but want to go back to the single mention of common sense in our target article. There, we discussed the advantage sometimes taken by *scientists* of commonsense beliefs.

Numerous MGB articles, reviews, and popular press pieces (as well as one of our commentaries) play on the phrase "gut

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feelings." Some elaborate why using this phrase is an appropriate vindication of ordinary thinking. For example, one MGB review suggests that "in daily life, we use common phrases such as 'butterflies in my stomach' or 'I have a gut feeling' to articulate our intuitions, the first warning signs when something is wrong. This concept that the gut can reveal, or even predict, our thoughts and feelings is one that is ingrained in our psyche and is now gaining the attention it deserves in the scientific literature" (Cowan et al. 2018, p. 1). They go on to say that such intuitions have something to do with the gut-brain-axis, which now includes the microbiota (hence MGB).

We fully understand how tempting catchphrases can be and why connections between "folk" uses of language might be made in scientific research areas. But there is seldom any straightforward relationship between folk use and scientific terminology, as detailed investigations of folk and scientific concepts such as "species," "innateness," "free will," and "causation" have shown (e.g., Griffiths 2002; Hunn 1976; Nichols 2004; Norton 2003). Even without thinking too hard, it should be obvious that whatever microorganisms are doing in our gut, these activities simply will not translate into "thoughts and feelings" in any comprehensible sense. Our main reason for urging caution with such appeals to commonsense terminology is because of the tendency to "oversell" microbiome research in general (Eisen 2017) and MGB in particular (as outlined in our target article). One of the temptations to oversell can come from appeals to commonsense notions, when these verbal vehicles may already come with strong and potentially incompatible perspectives and may simply not capture at all the phenomena the science is investigating.

For example, **Wissel & Smith** mention findings about "the microbiome of romantic couples" (para. 3) when they cite a recently published study (Dill-McFarland et al. 2019). It finds that married couples reporting "closeness" have more similar microbiomes than less close spouses, non-spouses, and even siblings. This article explains these correlations as attributable to sustained marital intimacies that presumably transfer microbes from one spouse to the other. Caregiving relationships that involve personal hygiene probably achieve similar effects. But by reporting this study with the label "romantic" (never mentioned in the cited paper), and then immediately invoking a microbiome-neurotransmitter correlation from another paper, Wissel & Smith might be thought to offer up these findings to the very sensationalism they criticize. We expect to read headlines any day now saying, "Microbiomes responsible for romance!"[1] One of the points in our target article was that scientists too have to take responsibility for their language and interpretations, and talking about "romance" rather than close cohabitation effects on microbiome composition is not going to help the field's relationship with media.

Clark partly defends the use of some catchy phrases saying that "many playful, flashy, even personifying interpretations and labels, now common to modern-day multimedia presentation styles, enrich the public's imagery of difficult-to-learn science concepts" (para. 3). Although we think this can be justified in appropriate contexts, there is also a very fine line between finding good metaphors to communicate science to non-specialists and over-simplification accompanied by false promises. **Borghi et al.** notice "pressure towards public engagement for professionals without specific training in science communication" (para. 1). There is a whole academic discipline of science communication out there – in most universities, in fact – and working with this discipline might be valuable for MGB research, scientific groups in general, and the future of science.

R4.3. Terminology

Being careful with language is not just about wording, but also conceptualization and operationalization. **Birk** urges MGB and other researchers to be more precise about the use of the term stress. He locates several definitions in our sample of highly cited MGB papers and finds "no coherent theoretical paradigm and … very different methods of inducing 'stress'" (para. 2). Our target article noted efforts to avoid anthropomorphizing of anxiety and depression, yet this does not happen with "stress," observes Birk. He gives very good examples of problems with the unqualified use of this term, which occurs well beyond the MGB area of research. We greatly appreciated his concluding salvo: that rather than grand discussions of microbiomes and their impact on the human self (as is done in Rees et al. 2018), the field needs to deflate itself to deal with basic operationalization of terms.

Birk also shows that "social" is another inconsistently deployed concept in rodent behavioural tests. He goes on to argue more generally that all scientific terms and concepts need careful operationalization. We agree wholeheartedly. Our pet peeve in MGB research and beyond is the term "dysbiosis." **Johnstone & Cohen Kadosh**, for example, take the term to be part of established microbiome explanations. For them, "dysbiosis has been shown to be related to symptoms of anxiety and depression," and that there are "consequences of dysbiosis [that are] particularly critical during development" (para. 3). They suggest that improved MGB research can be achieved simply by taking into account "the *timing* of dysbiosis" (para. 3). **Andreoletti & Rescigno** likewise believe that dysbiosis contributes to disease, and that it is a generic phenomenon untroubled by the inter-individual variability of microbiomes.

As we outlined in our target article and have addressed in earlier work (Hooks & O'Malley 2017), the term *dysbiosis* suffers from loose and messily overlapping definitions that often tie disease diagnosis and explanation together in a vicious explanatory circle. By throwing the term around so liberally (in the broader microbiome field, as well as MGB), causality is further obscured. Rather than talking about mere "changes" or "reduced diversity" in microbiomes as drivers of disease (e.g., **Johnstone & Cohen Kadosh**), researchers would be considerably better off specifying which fine-grained taxa are increasing or decreasing, their potential or actual roles in the causal pathways that lead to disease outcomes, and whether many of the microbes might simply be along for the causal ride when the host is diseased. All of these aspects of host-microbiome relationships are beginning to be addressed in broader microbiome research but have some way to go.

Alberts et al. try to avoid the conceptual issues of dysbiosis by referring to microbiomes as "dysregulated" instead of "dysbiotic." **Borghi et al.** refer coyly to "unbalanced ratios" of microbiota composition. Do these rewordings avoid the problem? We do not think so. First, there is no evidence that the microbiome should be treated by the host as a unitary entity that can be regulated to achieve a definitive desirable state rather than less desirable ones. Microbes come and go and compete furiously among themselves and with the host (Foster et al. 2017). Discussing stable states as "homeostasis" (the supposed opposite of dysbiosis) misleads about the dynamics producing such states. Although there may be microbiome states that have specific pathogens

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that affect host health, it is simpler and more accurate to refer to those taxa. If the microbiota is influenced by diet, and the diet seems to have effects on both host and microbiome composition, it is more straightforward to say just that. As for balance, this is the revival of an antiquated notion of thinking about health, and there is neither evidence nor theory to warrant claims about microbiome imbalance as a mechanism for disease (Olesen & Alm 2016).

When referring to "dysbiotic microbiota," **Fetissov** argues for a focus on "specific bacteria in modulating brain function and behaviour" (para. 1). This, we suggest, identifies very nicely where the true problems of the term dysbiosis are located along with their potential solutions. Although attempts have been made to identify "dysbiotic" signatures of whole microbiomes, ultimately, very particular groups of microbes are probably producing the harmful effects on the host (as **Lynch** notes, this is a more "proportionate" causal explanation than the whole microbiome). In such a situation, there is no need to mention "dysbiosis." Instead, it will be sufficient to specify which organisms are doing what and how these relationships hold under distinct (experimental) conditions. Ultimately, the issues to do with dysbiosis come back to the causal uncertainty of microbiome research, which has come up repeatedly in these three themes and the commentaries grouped under them.

R5. Core conclusions

We identified three major themes in the commentaries on our target article (Table 1). However, another way to categorize them is by *dimensionality* of the field and the *causal nature* of its findings.

Dimensionality refers to the calls made in multiple commentaries for more depth and scope of methods, so that MGB (and probably microbiome research more broadly) can understand microbiota across different populations, circumstances, and timescales. Although sometimes adding extra dimensions can simply look like throwing more methods or data at a problem, if we interpret this advice in the constructive sense it is offered, these commentaries have the much more justifiable aim of trying to gain closer insights into microbiome diversity in order to explain their effects on brain and behaviour more precisely.

But to explain any phenomenon is ultimately to achieve causal insight into it, and this is the second core demand of the commentaries. They suggest how to address causality: from specific angles (e.g., particular causal entities; function-based accounts) and within broader frameworks (e.g., complex causal networks; criteria of causal explanation). Most of our commentators see more robust causal attributions as the mandatory next step forward for MGB and microbiome research in general.

We noted in our target article how MGB is salutary in its focus on gaining experimental insights into connections between the microbiome and the brain rather than accumulating yet more high-throughput data. We applauded how these insights may eventually lend themselves to causal interpretations. What we questioned, and many of our commentators have now echoed and elaborated, is how justified many current causal interpretations are. We are delighted to see such a strong consensus on this core issue and look forward to seeing the different ways in which stronger causal explanations are realized in MGB research and microbiome studies more broadly.

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Note

1. It turns out we are a bit late on the uptake here. Jonathan Eisen already bestowed in 2015 an "Overselling the Microbiome Award" on a slew of microbiome-kissing-romance claims. See https://phylogenomics.me/2015/02/15/overselling-the-microbiome-award-cbc-fresh-air-ja-tetro-on-kissing-microbiomes/.

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[The letters "a" and "r" before author's initials stand for target article and response references, respectively]

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