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Microbiota-gut-brain research: a critical analysis

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Abstract
Microbiota-gut-brain (MGB) research manipulates gut microbes ('microbiota') in order to explore the connections between human gut microbiota and cognition, mood, and neurodevelopmental and psychiatric disorders. This growing body of research proposes new explanations of mental health and potential avenues of treatment. Limited critical attention has been paid to MGB, and this is ill-balanced by considerable scientific and public interest. We analyse the core methods and findings of MGB research, then reflect critically on their broader interpretations. Insight into current limitations provides a more realistic picture of the field to those outside it, and indicates how research can develop.

Keywords
Anxiety; Autism; Depression; Cognition; Gut-Brain Axis; Microbiome; Microbiota; Probiotics

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., Mayer et al. 2014; Liu 2017). 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. They 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 (Hanage 2014; Bik 2016; Quigley 2017), and MGB research in particular (e.g., Forsythe et al. 2016; Bruce-Keller et al. 2018), a systematic scrutiny from outside the field has yet to be achieved.

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 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' had to 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 (Renaud & Miget 1930; Evans et al. 1948). Although these effects were thought to be due to a diminution of "exudation and diapedesis of leucocytes" (Evans et al. 1948), it was realized much later that adrenaline also reduces the bactericidal activity of leukocytes (Qualliotine et al. 1972). Other experiments revealed that adrenaline administration actually reduces host mortality after the injection of bacterial toxins (Chedid & Boyer 1953; Hodoval et al. 1968), which suggested that this catecholamine has different effects on living bacteria. And although acetylcholine is known as the prototypical effector of the parasympathetic nervous system, an additional line of research showed that it can be produced by a strain of *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., *Gastroenterology* 1980; Aziz & Thompson 1998). A variety of important findings emerged about gut microbes, their cell wall components, and nervous systems or behavioural states (Hart 1988; Bluthé et al. 1992; Lyte 1993). Further extensive experimentation on the catecholamines, 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 (Tsavkelova et al. n.d.; Asano et al. 2012). 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 both the perspective of individual pathogens and more systemic community effects (see Haenel 1961; Savage 2001). While work in the 1980s had begun to examine mechanistically how specific intestinal microorganisms might affect mammalian brain states (e.g., Jeppsson et al. 1983; Brown et al. 1990), 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 the 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 Section 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 germfree (GF) mice. These are mice that are born and live their lives without microorganisms until they are experimentally colonized; other germfree 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 below), they have become the gold experimental standard for causal claims in human microbiome research, which now includes gut-brain studies.

Despite all 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 used germ-free mice to reveal that “commensal microbes [are] affecting the neural network responsible for controlling stress responsiveness” (Sudo et al. 2004, 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 germfree mice allow most of the complexities of microbiomes to be ignored: mice either have microbiota or they do not. Both this paper and earlier efforts 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 microbiome analyses were also included: the difficulty of identifying

causal pathways and yet the tendency to suggest microbiota were bring 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 detecting 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 (Park et al. 2013; Jiang et al. 2015), anxiety (Neufeld et al. 2011a; Crumeyrolle-Arias et al. 2014), autism (Hsiao et al. 2013; de Theije et al. 2014), schizophrenia (Severance et al. 2016), post-traumatic 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 in order 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 Section 7).

An outline of some key studies in MGB research will help to 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 germ-free (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 administration of probiotic bacteria (*Bifidobacterium infantis*). Because this occurred only to the nine-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 Section 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 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. (2011) 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'), 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 (2011) 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. 2011, 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 afferents (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 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, due to hypothesized developmental timing. *B. fragilis* was known to improve gut effects from earlier immunological studies (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, while still incomplete, hint at the potential mechanistic pathways that might underlie microbiota effects on brain and behaviour.

Many MGB studies, including those above, are believed to be relevant to human psychiatric disorders. But 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 conditioning 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-signaling 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 Supplementary Material, Section 1). 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 Section 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., Mayer et al. 2014; Liu 2017). And yet much of the research is highly speculative regarding mechanisms, some of it is contradictory, and many well-established methods are used in limited, mistaken, and even outdated ways, as we will show below.

Although some scientific papers and popular essays have already pointed toward central problems for microbiome research (e.g., Shanahan & Quigley 2014; Eisen 2017) and warnings have been issued about MGB ‘hype’ in particular (see comments in Zimmer 2014; Smith 2015), these discussions have not been based on detailed examinations of 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 external analysis of the field for anyone with interests in understanding human minds and behaviours, and their putative microbiome connections.

4. 25 most cited MGB papers

In order to analyse the field more closely, we examined the most highly cited 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: 25/05/2017; updated 11/07/2018). 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 nine highly cited articles. Finally, we conducted a third search using the more relaxed term of "brain microbiota". This search found 867 articles. We inspected the most cited articles of this group, which revealed another three 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 (Section 5) and behavioural tests and statistics (Section 6).

Table 1: 25 most cited papers in MGB research.
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.

Publication	**Citations**
Diaz Heijtz et al. (2011) Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci USA*	1348
Bravo et al. (2011) Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci USA*	1218
Hsiao et al. (2013) Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell*	1173
Sudo et al. (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol*	935
Bercik et al. (2011) 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. *Biol Psychiatry*	613

Neufeld et al. (2011b) Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterol Motil*	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. *Br J Nutr*	594
Clarke et al. (2013) The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol Psychiatry*	507
Bailey et al. (2011) Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain Behav Immun*	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 Behav Immun*	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 Behav Immun*	211
Leclercq et al. (2014) Intestinal permeability, gut-bacterial dysbiosis, and behavioral markers of alcohol-dependence severity. *Proc Natl Acad Sci USA*	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 Behav Immun*	166
Bruce-Keller et al. (2015) Obese-type gut microbiota induce neurobehavioral changes in the absence of obesity. *Biol Psychiatry*	161
Desbonnet et al. (2015) Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain Behav Immun*	159
Neufeld et al. (2011a) Effects of intestinal microbiota on anxiety-like behavior. *Commun Integr Biol*	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. *Neurogastroenterol Motil*	131
Park et al. (2013) Altered colonic function and microbiota profile in a mouse model of chronic depression. *Neurogastroenterol Motil*	130

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 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 a methodological primer and update). 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 amount of the MGB research in our top-cited sample does not even achieve the cataloguing step.

The gut is the most studied but also the most complex human-associated microbiome. 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 (Momozawa et al. 2011; Gevers et al. 2014; 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., Sudo et al. 2004; Gareau et al. 2011; Crumeyrolle-Arias et al. 2014). Sometimes a rescue experiment is performed in which a standard microbiota is transplanted into GF animals to investigate whether the phenotype will be reversed (Diaz Heijtz et al. 2011; Neufeld et al. 2011a, 2011b; Clarke et al. 2013).

2) Studies of normally colonized mice treated with antibiotics (Bercik et al. 2011; Ait-Belgnaoui et al. 2012; Bajaj et al. 2013; Desbonnet et al. 2015). One study in our sample then used re-colonization of the animals with bacterial flora 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 Material, Table 2).

4) Standard microbiota studies that assess the experimental alteration of gut flora (Supplementary Material, 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 Material, 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 WHO definition of a probiotic: any live microorganism that is used to intervene in a human body and which can 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 "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 Methods, 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). While 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 Section 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 Section 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), since 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). Yet all 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 Section 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 (Weiss et al. 2016; Knight et al. 2018). For example, one of the reasons that standard parametric tests are not adapted to microbiome data is the issue of compositionality. Rapid changes to any single taxon in the microbiota are often measured as changes to all 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 Material, 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 glucocorticoids

(Supplementary Material, Table 4a). All 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 Material, 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 Material, Table 4c). Only two studies present work on animal models of autism spectrum disorder (Supplementary Material, Table 4d), and six address different forms of cognition (Supplementary Material, Table 4e).

Most of the studies we examined do not explicitly justify their methodologies. They seldom address the potentially confounding effects (e.g., maternal separation, 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 examines corticosterone in the context of stress – a framework 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 circulating concentration of this glucocorticoid (Wang et al. 1999; Toda et al. 2004). 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 stucture and neuroanatomy, different microbiota, and different evolved behaviours (see Nguyen et al. 2015; Arrieta et al. 2016), 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, 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', 'depression-like'), we nevertheless found several instances of terms for multidimensional human disorders (e.g., 'anxiety', 'depression') being applied to the unidimensional rodent results (see Supplementary Material, 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 behavioral despair that is indicative of human depression-like behaviour (Molendijk & de Kloet 2015; Commons et al. 2017).

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) 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 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., Goehler et al. 2008; Bassi et al. 2012), 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 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 Material, Table 6). In particular, one-way ANOVAs or Student’s t-tests are frequently employed when the experimental design includes more than one independent variable. In such cases, two- or three-way ANOVAs are required (e.g., Ait-Belgnaoui et al. 2012; 2014; Ohland et al. 2013). In many biological situations, the effect of one factor on an outcome of interest often depends on other factors. Thus, 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 as well as 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 Material, 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 above, “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 guidance.

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 may come.

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., Bravo et al. 2011; Ait-Belgnaoui et al. 2014) – are offset by more conservative elaborations, sometimes in the very same paper (e.g., Foster & McVey Neufeld 2013; Christian et al. 2015). 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 40-50%; see Supplementary Material, Section 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., Rosen & Palm 2017; O'Malley & Skillings 2018).

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. 2011; 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., O’Mahony et al. 2009; Crumeyrolle-Arias et al. 2014; Bruce-Keller et al. 2015). 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., Shanahan & Quigley 2014; Olesen & Alm 2016; Hooks & O’Malley 2017; 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. 2011; Neufeld et al. 2011). 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?

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., Sudo et al. 2004; Bailey et al. 2011). 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 wiped out 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 thus 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* have evolved to infect cats via rodents, and so the former 'manipulate' 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 the 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.

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 leads 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 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.

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 due 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 (Dupont et al. 1965; Braniste et al. 2014). 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 hinting 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 were, 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. 2017). 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.

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; Steenbergen et al. 2015; Slykerman et al. 2017; 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 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 (McNulty et al.

2011; Kristensen et al. 2016). Sampson and Mazmanian (2015) account for the absence of evidence by suggesting more indirect attributions: "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 above). They are very difficult claims to test, especially in a medical context. One of our most-cited MGB papers, Ohland et al. (2013), 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 Supplementary Material, Section 7). Commercial investment in probiotics is increasing (Olle 2013; Jabr 2017), as is academic patenting activity related to probiotic and other microbiota-based therapies (Supplementary Material, Figure 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, comes in fourth (Supplementary Material, Figure 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 above will have limited appeal. However, as some commentators have noted (e.g., Olle 2013), focusing on a few classic probiotic strains – identified over 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 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.

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 searches still yield more than 300 press publications

(see Supplementary Material, Section 8, 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 2015). 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 2015).

A valuable lesson for press releases about research can be learned from associations found early in microbiome research history about 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 (Sze & Schloss 2016; Duvallet et al. 2017). Initial findings, although widespread, were from small samples, with hidden variations in background conditions (Schloss 2018). As we already noted, high inter-individual variablility 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; Gonon et al. 2012). While 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 amount 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 Material, Table 8). Additionally, we performed a survey of Twitter posts of news articles in 2017 (see Supplementary Material, 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’ approach (Fleming 2017; many reader comments are skeptical 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, more fibre; get more exercise and 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. Because clear cause-effect links between diet-altered microbiota composition and bodily or mental status are unknown, these gaps leave room for the sentiment that it's 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 (Rao & Gershon 2016; Quigley 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? Won't it 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 on-going 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 on-going need for more connected research that is able to investigate the complex interactions occurring in multipathway systems. Expecting magic bullets of treatment to emerge from these early days in which 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, this 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.

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 Section 2), and if worked 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.

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., Lyte 2011; Bruce-Keller et al. 2018; Schloss 2018). More sophisticated microbiota sampling and analysis will help 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.

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 Section 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.

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