MICROBIAL ECOLOGY in Health and Disease

COACTION PUBLISHING

THE MICROBIOME IN AUTISM SPECTRUM DISORDER

Towards large-cohort comparative studies to define the factors influencing the gut microbial community structure of ASD patients

Daniel McDonald[1,2], Mady Hornig[3,4], Catherine Lozupone[5], Justine Debelius[2,6], Jack A. Gilbert[7,8,9,10]* and Rob Knight[2,6,11]

[1]BioFrontiers Institute, University of Colorado, Boulder, CO, USA; [2]Department of Computer Science, University of Colorado, Boulder, CO, USA; [3]Center for Infection and Immunity, Columbia University Mailman School of Public Health, New York, NY, USA; [4]Department of Epidemiology, Columbia University Mailman School of Public Health, New York, NY, USA; [5]Department of Medicine, University of Colorado Denver, Anschutz Medical Campus, Aurora, CO, USA; [6]Department of Chemistry and Biochemistry, University of Colorado at Boulder, Boulder, CO, USA; [7]Institute for Genomic and Systems Biology, Argonne National Laboratory, Argonne, IL, USA; [8]Department of Ecology and Evolution, University of Chicago, Chicago, IL, USA; [9]Marine Biological Laboratory, Woods Hole, MA, USA; [10]College of Environmental and Resource Sciences, Zhejiang University, Hangzhou, China; [11]Howard Hughes Medical Institute, Boulder, CO, USA

Differences in the gut microbiota have been reported between individuals with autism spectrum disorders (ASD) and neurotypical controls, although direct evidence that changes in the microbiome contribute to causing ASD has been scarce to date. Here we summarize some considerations of experimental design that can help untangle causality in this complex system. In particular, large cross-sectional studies that can factor out important variables such as diet, prospective longitudinal studies that remove some of the influence of interpersonal variation in the microbiome (which is generally high, especially in children), and studies transferring microbial communities into germ-free mice may be especially useful. Controlling for the effects of technical variables, which have complicated efforts to combine existing studies, is critical when biological effect sizes are small. Large citizen-science studies with thousands of participants such as the American Gut Project have been effective at uncovering subtle microbiome effects in self-collected samples and with self-reported diet and behavior data, and may provide a useful complement to other types of traditionally funded and conducted studies in the case of ASD, especially in the hypothesis generation phase.

Keywords: *autism*; *microbiology*; *microbiome*; *neurological*

Responsible Editor: Richard E. Frye, Arkansas Children's Hospital Research Institute and University of Arkansas for Medical Sciences, USA.

*Correspondence to: Jack A. Gilbert, Argonne National Laboratory, Biosciences Division, B202 Room B363, 9700 S Cass Avenue, Lemont, IL 60439, USA, Email: gilbertjack@anl.gov

This paper is part of the Supplement: *The Microbiome in Autism Spectrum Disorder*. More papers from this supplement can be found at http://www.microbecolhealthdis.net

Received: 4 November 2014; Revised: 12 February 2015; Accepted: 12 February 2015; Published: 9 March 2015

Differences in the gut microbiota that inhabit the intestinal tracts and feces of children with autism spectrum disorders (ASD), as compared to neurotypical children, have been reported by several research groups over the past decade (1–5) [for comprehensive review, see (6)]. The relationship of these differences in the microbiota to dietary practices, the diversity and severity of clinical features, and pathogenesis remains unclear. There is now evidence in animal models (1, 7) as well as from more limited studies in humans, that signaling along the gut-microbiome–brain axis is a critical regulator of both central nervous system and immunefunction (8, 9). In addition, some studies suggest that interventions targeting the microbiome (probiotics, fecal transplants) may have utility in the management of other neuropsychiatric disorders (9–15). Further research to delineate the extent of involvement of gut microbes in autism, and to monitor or even suggest therapies, is therefore promising.

The role of bacteria co-associating with our gastrointestinal tract in physiological development and disease

Citation: Microbial Ecology in Health & Disease 2015, **26**: 26555 - http://dx.doi.org/10.3402/mehd.v26.26555

has recently attracted considerable attention, primarily as a result of technological advances associated with sample processing, sequencing of genetic information, and data analytical tools. In the last decade, the revolution in sequencing technology has fundamentally altered our perception of microbial diversity and ecology, by enabling us to process and analyze thousands to tens of thousands of samples in a single study (16–18). These advances have allowed us to identify significant trends relating the physiology, environment, and health history of a host and the presence or relative abundance of the bacteria that inhabit the host (e.g. 19–21). Many factors affect the colonization and succession of the microbial communities that live within us, and that change over time (22, 23). It is therefore difficult to capture the combination of events within an individual's life that have resulted in that individual's unique microbial signature. Although some bacterial taxa correlate strongly with specific conditions (19, 24, 20, 21), other relationships are less obvious, and may require far larger cohorts of participants to detect (16).

Bacteria have profound influences on key aspects of our immune regulatory network (25), with far-reaching implications for our physiological and even neurological development (26). Direct association between bacteria and host cells is important for immunological development (27), regulation, and response (28). However, bacterial biomass in the lumen, including bacteria that do not actively associate directly with host gastrointestinal tissues, might be more important for the production of key metabolites that can have important physiological effects once they cross into our bloodstream [e.g. 4-ethyl phenyl sulfate (4-EPS) production (1)]. Bacteria contribute to circulating blood levels of amino acids such as tryptophan (including synthesis from dietary serine or indoles), thereby affecting levels of key regulatory neurotransmitters, such as serotonin, and also regulate levels of neuroactive metabolites along the tryptophan degradation (kynurenine) pathway both in the intestine and in the blood (8, 29–31). Although the common method for assessing a gut microbial community is through the feces, in some circumstances such as inflammatory bowel disease (IBD), mucosal biopsies may help identify bacterial associations that may not be evident in fecal samples, especially in cases where mucosally associated bacteria are not dominant in the fecal sample (32).

The importance of experimental designs: cross-sectional versus longitudinal analysis

Given the heterogeneity of ASD and the many potential confounding factors that may influence microbial diversity, looking for associations in very large and well-characterized cohorts may be the key to finding associations between the gut microbiota and disease. Large-scale efforts such as the Earth Microbiome Project (17) and American Gut (http://americangut.org) have demonstrated the willingness of large communities of researchers, and even of the general public, to contribute thousands of samples to provide a fuller picture of the microbial diversity of our planet and our bodies. In particular, aggregating longitudinal datasets from different microbial habitats is starting to provide an understanding of dynamics on different timescales (33), and extending these to studies of people with different clinical conditions provides an especially exciting opportunity at present.

Cross-sectional study designs

Cross-sectional study designs are useful for identifying systematic patterns across a population, testing the hypothesis that some component of microbial variation within a population is correlated with a study parameter (e.g. ASD diagnosis). Applying a cross-sectional study design to very large cohorts, for instance thousands of subjects, may provide the statistical power to elucidate subtle phenomena when faced with many confounding factors, as is common in microbiome studies where lifestyle, diet, age, genetics, and disease play important roles in shaping community structure.

The benefit of a large cross-sectional study design was demonstrated during an early analysis of the American Gut dataset (http://americangut.org). At first, patterns driven by diet and other lifestyle choices were observed, but statistical significance suffered from limited sample sizes within the specific groups of subjects showing interesting trends. As we collected thousands of additional samples, many of these groups reached sample sizes that increased the confidence and significance of the observed patterns. One such pattern was a population-scale seasonal effect, in which samples collected from individuals during the holiday season in the United States tended to have higher diversity within each sample (http://nbviewer.ipython.org/github/biocore/American-Gut/blob/master/ipynb/Alpha diversity notebook.ipynb). Empirical power estimations suggest around 100 samples per group are required to reliably observe these subtle differences across seasons, even after matching individuals for a variety of other factors (http://nbviewer.ipython.org/github/biocore/American-Gut/blob/master/ipynb/Power.ipynb). These more subtle patterns only appeared through the collection of a large number of samples from a broad cross-section of the population, making it possible to detect the signal against high levels of background noise coming from other factors.

Another recent microbiome study that focused on Crohn's Disease patients (32) and relied on a large cross-sectional design also benefitted greatly from a large sample size. Critically, the researchers noted that the number of samples was more important than sequencing depth (the number of sequences collected from each sample) for detecting statistically significant patterns that were apparent in the full dataset. The study design allowed conclusive identification of key taxa that differentiate Crohn's

Citation: Microbial Ecology in Health & Disease 2015, **26**: 26555 - http://dx.doi.org/10.3402/mehd.v26.26555

patients from healthy controls that had not previously been reported as associated with Crohn's. Interestingly, once the specific taxa were identified, it was then possible to assess whether the metabolic potential of these organisms made sense in the context of the disease. In this case, some of the microbes that are less abundant in Crohn's patients are involved in the production of butyrate, which is a short-chain fatty acid (SCFA). Butyrate is consumed by intestinal epithelial cells (34), which are instrumental in initiating an immune response (35, 36). In addition, the researchers were able to identify an amplification effect from antibiotic usage, in which individuals who had recently taken antibiotics had a significantly pronounced increase in detrimental taxa observed in Crohn's patients, with a corresponding decrease in beneficial taxa. One taxon in particular, *Fusobacterium*, was recently found to be highly correlated with colorectal cancer (37), which has a higher incidence in Crohn's and IBD patients. These observations suggest antibiotic usage by this population should attract closer scrutiny due to the increased risk to the patient [although it should be noted that the specific effects of antibiotic usage in healthy individuals is still poorly understood, and appears to be highly variable in different subjects (38, 39)]. A parallel study in ASD, especially one relating differences in the microbiota to common interventions such as drugs targeted at resolving gastrointestinal symptoms, antipsychotics, antidepressants, dietary changes, and other treatments, and with excellent clinical data, could be especially valuable in understanding which changes in the microbiome are likely to be associated with ASD symptoms and which are most likely to be side-effects of treatment.

Longitudinal study designs

Although cross-sectional studies are useful, they cannot provide insight into variation within an individual over time, limiting their power to observe phenomena such as succession and to factor out between-subject variation in diseases with complex etiologies. Such questions can only be addressed with longitudinal study designs, examining multiple timepoints from the same individual. Ecological succession of the gut microbial community is of particular interest in autism because microbial communities play a central role in training the immune system during childhood development (22). Early antibiotic usage, for example, is associated with an increase in allergies and obesity (40, 41), and may be associated with disrupting the maturation of the microbiome. Within the human microbiome, an infant's initial microbial communities depend on delivery mode (42), where the infant fecal community tends to resemble the mother's vaginal community after vaginal birth, but instead resemble skin after C-section. Koenig et al. (22), through a 3-year time series tracking a newborn, monitored this succession, revealing a large amount of change over time progressing from a vaginal-like community to a community resembling the adult fecal state (43). One particularly interesting observation was a substantial regression in community state as a result of the child receiving antibiotics. This regression was rapidly ameliorated, suggesting that resilience in the community is picked up relatively early in life. However, the impact that these types of disruptions can have on the fledgling immune and endocrine systems is not yet known, nor is the magnitude of this impact with respect to other environmental, dietary, and lifestyle factors.

Some important general considerations in longitudinal study designs include how frequently to sample, whether to focus timepoints around defined interventions, and what auxiliary data (e.g. diet or immunological data) need to be collected at each timepoint versus assessed once for each subject. In general, not enough studies have been done in order to provide detailed guidance on these points, and animal model studies can be misleading. For example, on the basis of studies on mice, which respond within 1 day to dietary shifts (44, 45), we performed a parallel dietary intervention study in humans with very little effect after 10 days in an inpatient setting (46). However, longitudinal studies of the effects of microbiota transfer from humans to mice have been very useful for elucidating effects of microbiomes associated with obesity (47) and malnutrition (48), and the same is likely to be true for autism (49) given the availability of mouse models (1). Given the established role of gut microbiota in allergen sensitization in mouse models (28), and given high variability among individual animals as well as among individual humans, understanding effects of changes in the microbiome in response to defined perturbations is likely to benefit considerably from animal model work even when details of the timescale or nature of the response differ among species.

Longitudinal studies of the human microbiome to date have typically employed small sample sizes, limited timepoints, or both. For example, the NIH-funded Human Microbiome Project (16) reported data from only two timepoints in each of 250 subjects. Only a couple of daily studies of apparently healthy adults have employed sampling durations as long as a year (50, 51), and a recent study of dozens of healthy students employed only weekly sampling (52). Nonetheless, it is clear that dynamics are shaping up to be an important aspect of the human gut microbiome, and studies both of the baseline dynamics of the microbiome in ASD subjects, and of dynamics in response to treatment with different interventions aimed at alleviating different ASD symptoms, hold considerable potential both for stratified treatment and for developing a better biological understanding of the underlying mechanisms.

Comparison of study designs with respect to neuropsychiatric disorders

Within the context of neuropsychiatric disorders, cross-sectional designs have been instrumental in recognizing the correlation between the presence of blood markers of inflammation or intestinal barrier compromise and depression (53), bipolar disorder (54), and autism (6, 55). The pathogenesis of these diseases differs. However, the implication of inflammation in such a broad range of disorders suggests that inflammation, and both its cause and effect, ought to be a focal point for investigation. In particular, inflammation can lead to a permeable gut, thereby allowing metabolites produced by gut inhabitants (and even the inhabitants themselves, or fragments of them) to leak into the bloodstream (56, 57), and some metabolites can even pass the blood/brain barrier (58). On the other hand, the predominant source of serotonin in the body is within the large intestine, and it is the role of enterochromaffin cells to synthesize serotonin from tryptophan (8). Dysregulation of the gut microbiome can trigger secondary effects in these cells that alter the rate of serotonin production (59), with significant changes in neuropsychiatrically relevant domains, including mood (60) and satiety (61) and possibly, the stereotypic features of autism (62, 63). Interestingly, some of the classes of drugs prescribed for treatment of neuropsychiatric disorders act on the gastrointestinal tract and may also affect the immune system (59). One metabolite of interest is 4-EPS, originally observed to be significantly increased in serum in a mouse model of autism (1) [fascinatingly, this model requires stimulating the mother's immune system prior to birth, resulting in offspring with autism-like symptoms (64)]. Anorexia Nervosa is an eating disorder characterized by the inability or unwillingness to gain weight (65, 66). ASD is a comorbidity for anorexia, and may be reflective of sociocommunicative problems within individuals with anorexia (67–69). The microbiome plays a role in the pathology of anorexia; the bacterial ClpB heat shock protein can induce anti α-MSH antibody, leading to a reduction in appetite, weight loss, and anxiety (70, 71).

The importance of controlling for technical variables in traits with small effect size

The problem of large versus small effect sizes is in some ways analogous to assessing the risk of a campfire sparking a wildfire. If you asked: are campfires correlated with wildfires, the answer is likely to be yes by analysis of whether wildfires are more likely in proximity to campgrounds. A large amount of variation in the type of camp, its geographic location, and definition of wildfire can likely be tolerated. In this case, the presence of a fire is a large effect. If instead you asked: are certain personality types more likely to spark a wildfire from a campfire, then the answer is subtle necessitating finer control over data collection. For instance, how personality type is assessed is critical in assuring that everyone underwent the same test and that there was no researcher bias in test administration. In addition, controlling for substance use is necessary in order to understand whether it is personality, or say, the presence of alcohol that leads to accidental wildfires. In this case, the potential small effect of personality (which is a large effect in other contexts) requires more careful control, relative to the large effect of simply having a campfire, in order to properly identify if in fact there is an effect mediated by personality.

The complexity of neurological disorders, and the difficulty to date in pinpointing specific causes, suggests that the causes themselves are varied, subtle, and possibly multifactorial. As such, emphasis on controlling for technical variables is essential to minimize noise, and maximize signal. For instance, the Human Microbiome Project sequenced two separate regions of the 16S rRNA gene (16) from the same samples leading to a confounding effect if analyzing both loci together. The end effect was that it was not feasible to compare data from one loci to another as the noise stemming from the loci masked any usable signals in the data. On a practical level, using the exact same protocols for all samples of a common type is critical in order to limit the impact of technical bias. Frustratingly, there is even variation that is introduced into the data by the site that is processing the samples, though there are ongoing efforts to understand the drivers of this variation so that it can be normalized, something that is necessary for clinical applications of microbiome assays.

Digging deeper, in addition to tightly controlled technical variables and large sample sizes, using a tiered systems approach can substantiate interpretation and validation. This is particularly useful within studies of autism as there is evidence for genetic predispositions that may 'activate' through an environmental trigger, where the microbiome is considered part of the environment. The systems approach can greatly improve the understanding of the roles particular organisms are undertaking. From 16S rRNA data, it is possible to predict a likely functional metagenomic profile (72), but it is not feasible to predict the specific metabolites being produced, which will to a certain extent be modulated by the availability of fermentable substrates, and other sources of energy for microbes. These metabolites are the vector of communication between microbes, and between microbes and the host. Similarly, knowing the genetic makeup of the host is informative, but it cannot be used to determine a specific immune response. A tiered approach that includes immunological markers, metabolite profiles, and microbial community data sampled at near the same time point enables researchers to tightly validate observations between levels, and truly begin to understand the dynamics of disease.

Citation: Microbial Ecology in Health & Disease 2015, **26**: 26555 - http://dx.doi.org/10.3402/mehd.v26.26555

The influence of diet on the microbiome

Perhaps unsurprisingly, what one eats can influence the composition of the microbiome. Long-term diet has one of the largest known effects on the human gut microbiome: in particular, the balance of carbohydrates to animal protein affects the balance of *Prevotella* sp. to *Bacteroides* sp., driving the largest component of overall patterns in the human microbiome within healthy adult Western populations (46). Cross-culturally, societies with high-grain, low-animal diets also tend to have far more *Prevotella* at the expense of other major gut taxa (23).

Most short-term dietary changes have been far more modest. However, on the extreme end, shifting to a heavy animal product diet characterized by meats and cheese can, on very short time scales, increase abundance of bile-tolerant organisms (73). The increase in these organisms is negatively correlated with acetate and butyrate stemming likely from the reduced fiber load available for microbial fermentation. Butyrate has previously been observed to modulate colonic regulatory T-cell differentiation in murine models (74), and is of particular interest within the study of neurological disorders due to the observed relationships with gut inflammation. The role of dietary gluten and casein in the etiology of ASD remains of intense interest to the community, but strong evidence to date has been scarce [reviewed in (75)].

Propionic acid, a SCFA produced by the microbiome from fermentable dietary carbohydrates (76), has been associated with ASD in rat models (58). ASD-like symptoms, including a neuroinflammatory response, can be induced from intraventricular infusion of propionic acid (58) resulting in significant changes in behavior and social interaction (77). However, pathology might only occur in individuals with genetic and/or acquired aberrations in metabolism, since in healthy individuals SCFAs are primarily metabolized in the liver (76), again indicating that associations between gut microbiota and ASD may also involve other underlying genetic factors. In healthy individuals, propionic acid potentially can increase feelings of satiety, lower carcinogenesis and cholesterol (78) and possibly have an anti-inflammatory effect (79). See (78) for an in depth review of propionate, including a discussion on fermentable substrates.

The possible role of vitamin D in ASD is also intriguing. Dark/yellow skin requires increased ultraviolet (UV) B exposure to induce sufficient vitamin D in low sunlight regions/seasons (80). This effect is particularly accentuated among Somalian and other immigrants from sub-Saharan Africa with fundamentalist practices leading to full body cover (81). There are also dietary practices [avoidance of vitamin D-enriched dairy products, and other common staples such as maize (82)] that may exacerbate deficiencies in vitamin D relating to reduced UVB exposure, contributing to Th1 skew/altered intestinal inflammatory state (83) as well as frankly increased risk of some infectious diseases such as tuberculosis (81). In the context of vitamin D deficiency (and perhaps also with low levels of lithocholic acid), vitamin D receptor (VDR) expression should be increased (84, 85). VDR has been reported to negatively regulate intestinal NFkB (and therefore downstream innate immune signaling) induced by bacteria, and bacteria also regulate VDR expression (86). This may be an important mechanism for explaining the role of vitamin D deficiency in autoimmune diseases, perhaps through a Th17 mechanism involving bacteria that play roles similar to the regulatory roles that segmented filamentous bacteria (SFB) play in mice (87–89).

Although we have a limited understanding of how diet influences the microbiome in the short-term [through extreme changes (73)] and more long-term phenomena (90), we do not yet understand how to manipulate diet to guide a microbial community from one state to another (e.g. from disrupted to healthy). One aim of the American Gut Project is to characterize diet and its impact on the microbiome, with the hope of elucidating systematic differences – if they exist – between dietary restrictions (e.g. vegetarians and paleo eaters). Unfortunately, the reliability of dietary data collected from the general public is often low. Even the recall of individuals for meals consumed over the course of a week can be compromised (91). The first attempt at collecting detailed diet information by the American Gut Project yielded limited results (though a correlation in diversity with the number of different types of plants consumed was observed). Variables such as the approximate percentage of fat consumed over the course of a week had incredible variance and in many cases were outside of reality. In its second attempt at diet, the American Gut Project decided to take a two-pronged approach, one using a generalized diet questionnaire that lacked free text entry and contained questions about the frequency of consumption (e.g. in an average week, how often do you consume at least 2–3 servings of fruit in a day?), and the second to use a validated food frequency questionnaire through a professional service called Vioscreen.

Correlation versus causation

As noted above, several intriguing correlations have been observed between ASD and the gut microbiota. However, establishing causality is challenging. In particular, gut barrier dysfunction is a common comorbidity of ASD, but is known itself to affect the microbiome both in humans and mouse models. Therefore, appropriate controls need to be selected carefully so that the effects of ASD itself are not confounded with the effects of gut barrier dysfunction. Longitudinal studies can help resolve these types of issues, for example, by testing whether changes in the microbiota associated with ASD precede or follow gut barrier dysfunction issues within a subject, although very limited data are available at present.

In mouse models causality is easier to establish because symptoms that model ASD can be induced experimentally and the ability to administer microbially based therapies is substantially greater. The best example of this to date is the MIA study described above (1), wherein autism-like symptoms can be traced to specific metabolites produced by the microbiome, and even reversed using probiotics. For other conditions, including malnutrition (48) and obesity (47), causal pathways may be uncovered by transplanting microbes from humans with a different physiological state into mice and demonstrating that aspects of the phenotype can be recaptured, either using fecal samples directly or using large collections of strains of bacteria isolated from individual fecal specimens (the latter providing evidence that only the bacteria are involved, as metabolites, viruses, antibodies, etc. are not transferred in these experimental designs). These types of studies therefore hold considerable promise for unraveling causality in ASD (49).

Conclusion

The lifestyle and dietary choices of individuals affected by ASD span a broad range, complicating analyses. As was the case with Crohn's Disease, the ability to observed subtle, and informative patterns depends on large sample sizes that are only feasible in cross-sectional study designs. Longitudinal designs, on the other hand, offer perspective into the change of a community over time, allowing tests of hypotheses about factors leading to a change in state within an individual (e.g. is a measured parameter such as disease severity modulated by changes in the microbiome) and whether a change in the microbial community happens prior to observable changes in individual state (e.g. reported severity) or vice versa, allowing inferences about causality. As we learned with the American Gut Project, the general public is extremely interested in microbiome research at present, and providing appropriate mechanisms to engage the public is an effective means to get to sufficient sample sizes to have the power to detect subtle differences in the data. Longitudinal studies, due to the high level of dedication over an extended period, cannot reach comparable sample sizes due to their expense. Given that a large sample size is more difficult in these designs, strict exclusion criteria must be defined to minimize confounding factors, maximize the signal, and maintain a high probability that the individuals will continue to the end of the study.

Studies of associations between ASD and the microbiome have generated a number of intriguing hypotheses about how microbes could be involved in the etiology of ASD. However, there are many confounding variables, such as diet and gastrointestinal comorbidities, as well as technical variation among studies and background microbiome differences among cohorts, that complicate analysis. Several approaches are likely to be exceptionally valuable in resolving such complexities: 1) access to large, cross-sectional cohort studies that can help generate hypotheses about combinations of factors that may have a strong influence on ASD outcomes, particularly if interacting in a nonlinear way; 2) longitudinal studies that allow high inter-individual variability in the microbiome to be factored out yet provide data regarding associations with progression within each individual to be revealed, potentially helping to get closer to causal pathways; and 3) animal model research including microbiome transfers and administration of candidate probiotics that will facilitate rapid progress toward understanding whether the microbiome plays a causal or contributory role in some subsets of ASD.

Acknowledgements

This work was supported in part by the US Department of Energy under Contract DE-AC02-06CH11357. DM was supported by the Interdisciplinary Quantitative Biology program (NSF IGERT award 1144807). RK was supported by HHMI, NIH, and CCFA. JWD was supported by NIH. MH was supported by NIH (NS086122), SFARI, and the Jane Botsford Johnson Foundation. CL was supported by NIH (K01 DK090285) and the Emch foundation.

Conflict of interest and funding

The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

References

1. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell 2013; 155: 1451–63.
2. Wang L, Christophersen CT, Sorich MJ, Gerber JP, Angley MT, Conlon MA. Increased abundance of *Sutterella* spp. and *Ruminococcus torques* in feces of children with autism spectrum disorder. Mol Autism 2013; 4: 42.
3. Adams JB, Johansen LJ, Powell LD, Quig D, Rubin RA. Gastrointestinal flora and gastrointestinal status in children with autism – comparisons to typical children and correlation with autism severity. BMC Gastroenterol 2011; 11: 22.
4. Williams BL, Hornig M, Parekh T, Lipkin WI. Application of novel PCR-based methods for detection, quantitation, and phylogenetic characterization of Sutterella species in intestinal biopsy samples from children with autism and gastrointestinal disturbances. mBio 2012; 3: e00261–11.
5. Williams BL, Hornig M, Buie T, Bauman ML, Cho Paik M, Wick I, et al. Impaired carbohydrate digestion and transport and mucosal dysbiosis in the intestines of children with autism and gastrointestinal disturbances. PLoS One 2011; 6: e24585.
6. Hsiao EY. Gastrointestinal issues in autism spectrum disorder. Harv Rev Psychiatry 2014; 22: 104–11.
7. Foley KA, MacFabe DF, Kavaliers M, Ossenkopp K-P. Sexually dimorphic effects of prenatal exposure to lipopolysaccharide, and prenatal and postnatal exposure to propionic acid, on acoustic startle response and prepulse inhibition in adolescent rats: relevance to autism spectrum disorders. Behav Brain Res 2014; 278C: 244–56.

Citation: Microbial Ecology in Health & Disease 2015, **26**: 26555 - http://dx.doi.org/10.3402/mehd.v26.26555

8. O'Mahony SM, Clarke G, Borre YE, Dinan TG, Cryan JF. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav Brain Res 2015; 277: 32–48.
9. Hornig M. The role of microbes and autoimmunity in the pathogenesis of neuropsychiatric illness. Curr Opin Rheumatol 2013; 25: 488–795.
10. Sha S, Liang J, Chen M, Xu B, Liang C, Wei N, et al. Systematic review: faecal microbiota transplantation therapy for digestive and nondigestive disorders in adults and children. Aliment Pharmacol Ther 2014; 39: 1003–32.
11. Aroniadis OC, Brandt LJ. Fecal microbiota transplantation: past, present and future. Curr Opin Gastroenterol 2013; 29: 79–84.
12. Dinan TG, Cryan JF. Melancholic microbes: a link between gut microbiota and depression? Neurogastroenterol Motil Off J Eur Gastrointest Motil Soc 2013; 25: 713–9.
13. Logan AC, Katzman M. Major depressive disorder: probiotics may be an adjuvant therapy. Med Hypotheses 2005; 64: 533–8.
14. Vitetta L, Bambling M, Alford H. The gastrointestinal tract microbiome, probiotics, and mood. Inflammopharmacology 2014; 22: 333–9.
15. Foster JA, McVey Neufeld K-A. Gut–brain axis: how the microbiome influences anxiety and depression. Trends Neurosci 2013; 36: 305–12.
16. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature 2012; 486: 207–14.
17. Gilbert JA, Jansson JK, Knight R. The earth microbiome project: successes and aspirations. BMC Biol 2014; 12: 69.
18. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 2012; 6: 1621–4.
19. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci USA 2005; 102: 11070–5.
20. Chen Y, Yang F, Lu H, Wang B, Chen Y, Lei D, et al. Characterization of fecal microbial communities in patients with liver cirrhosis. Hepatol Baltim Md 2011; 54: 562–72.
21. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 2008; 57: 1470–81.
22. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, et al. Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci USA 2011; 108(Suppl 1): 4578–85.
23. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature 2012; 486: 222–7.
24. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol 2012; 13: R79.
25. El Aidy S, Dinan TG, Cryan JF. Immune modulation of the brain-gut-microbe axis. Front Microbiol 2014; 5: 146.
26. Julio-Pieper M, Bravo JA, Aliaga E, Gotteland M. Review article: intestinal barrier dysfunction and central nervous system disorders – a controversial association. Aliment Pharmacol Ther 2014; 40: 1187–201.
27. Chu H, Mazmanian SK. Innate immune recognition of the microbiota promotes host–microbial symbiosis. Nat Immunol 2013; 14: 668–75.
28. Stefka AT, Feehley T, Tripathi P, Qiu J, McCoy K, Mazmanian SK, et al. Commensal bacteria protect against food allergen sensitization. Proc Natl Acad Sci 2014; 111: 13145–50.
29. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, et al. The microbiome-gut–brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol Psychiatry 2013; 18: 666–73.
30. De Theije CG, Wopereis H, Ramadan M, van Eijndthoven T, Lambert J, Knol J, et al. Altered gut microbiota and activity in a murine model of autism spectrum disorders. Brain Behav Immun 2014; 37: 197–206.
31. Bercik P, Collins SM. The effects of inflammation, infection and antibiotics on the microbiota-gut–brain axis. Adv Exp Med Biol 2014; 817: 279–89.
32. Gevers D, Kugathasan S, Denson LA, Vázquez-Baeza Y, Van Treuren W, Ren B, et al. The treatment-naive microbiome in new-onset Crohn's disease. Cell Host Microbe 2014; 15: 382–92.
33. Shade A, Caporaso JG, Handelsman J, Knight R, Fierer N. A meta-analysis of changes in bacterial and archaeal communities with time. ISME J 2013; 7: 1493–506.
34. Roediger WE. Utilization of nutrients by isolated epithelial cells of the rat colon. Gastroenterology 1982; 83: 424–9.
35. Weng M, Walker WA, Sanderson IR. Butyrate regulates the expression of pathogen-triggered IL-8 in intestinal epithelia. Pediatr Res 2007; 62: 542–6.
36. Kim MH, Kang SG, Park JH, Yanagisawa M, Kim CH. Short-chain fatty acids activate GPR41 and GPR43 on intestinal epithelial cells to promote inflammatory responses in mice. Gastroenterology 2013; 145: 396–406.e1–10.
37. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host Microbe 2013; 14: 207–15.
38. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci USA 2011; 108(Suppl 1): 4554–61.
39. Maurice CF, Haiser HJ, Turnbaugh PJ. Xenobiotics shape the physiology and gene expression of the active human gut microbiome. Cell 2013; 152: 39–50.
40. Chen Y, Blaser MJ. Inverse associations of Helicobacter pylori with asthma and allergy. Arch Intern Med 2007; 167: 821–7.
41. Cox LM, Yamanishi S, Sohn J, Alekseyenko AV, Leung JM, Cho I, et al. Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell 2014; 158: 705–21.
42. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci USA 2010; 107: 11971–5.
43. Lozupone CA, Stombaugh J, Gonzalez A, Ackermann G, Wendel D, Vázquez-Baeza Y, et al. Meta-analyses of studies of the human microbiota. Genome Res 2013; 23: 1704–14.
44. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med 2009; 1: 6ra14.
45. Crawford PA, Crowley JR, Sambandam N, Muegge BD, Costello EK, Hamady M, et al. Regulation of myocardial ketone body metabolism by the gut microbiota during nutrient deprivation. Proc Natl Acad Sci USA 2009; 106: 11276–81.
46. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science 2011; 334: 105–8.

47. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, et al. Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science 2013; 341: 1241214.
48. Smith MI, Yatsunenko T, Manary MJ, Trehan I, Mkakosya R, Cheng J, et al. Gut microbiomes of Malawian twin pairs discordant for kwashiorkor. Science 2013; 339: 548–54.
49. Gilbert JA, Krajmalnik-Brown R, Porazinska DL, Weiss SJ, Knight R. Toward effective probiotics for autism and other neurodevelopmental disorders. Cell 2013; 155: 1446–8.
50. Caporaso JG, Lauber CL, Costello EK, Berg-Lyons D, Gonzalez A, Stombaugh J, et al. Moving pictures of the human microbiome. Genome Biol 2011; 12: R50.
51. David LA, Materna AC, Friedman J, Campos-Baptista MI, Blackburn MC, Perrotta A, et al. Host lifestyle affects human microbiota on daily timescales. Genome Biol 2014; 15: R89.
52. Flores GE, Caporaso JG, Henley JB, Rideout JR, Domogala D, Chase J, et al. Temporal variability is a personalized feature of the human microbiome. Genome Biol 2014; 15: 531.
53. Maes M, Kubera M, Leunis J-C, Berk M. Increased IgA and IgM responses against gut commensals in chronic depression: further evidence for increased bacterial translocation or leaky gut. J Affect Disord 2012; 141: 55–62.
54. Severance EG, Gressitt KL, Yang S, Stallings CR, Origoni AE, Vaughan C, et al. Seroreactive marker for inflammatory bowel disease and associations with antibodies to dietary proteins in bipolar disorder. Bipolar Disord 2014; 16: 230–40.
55. Kang V, Wagner GC, Ming X. Gastrointestinal dysfunction in children with autism spectrum disorders. Autism Res Off J Int Soc Autism Res 2014; 7: 501–6.
56. Turner JR, Buschmann MM, Romero-Calvo I, Sailer A, Shen L. The role of molecular remodeling in differential regulation of tight junction permeability. Semin Cell Dev Biol 2014; 36: 204–12.
57. Berg RD. Bacterial translocation from the gastrointestinal tract. Trends Microbiol 1995; 3: 149–54.
58. Thomas RH, Meeking MM, Mepham JR, Tichenoff L, Possmayer F, Liu S, et al. The enteric bacterial metabolite propionic acid alters brain and plasma phospholipid molecular species: further development of a rodent model of autism spectrum disorders. J Neuroinflammation 2012; 9: 153.
59. Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG. The probiotic *Bifidobacteria infantis*: an assessment of potential antidepressant properties in the rat. J Psychiatr Res 2008; 43: 164–74.
60. Flory JD, Manuck SB, Matthews KA, Muldoon MF. Serotonergic function in the central nervous system is associated with daily ratings of positive mood. Psychiatry Res 2004; 129: 11–9.
61. Voigt J-P, Fink H. Serotonin controlling feeding and satiety. Behav Brain Res 2015; 277: 14–31.
62. McDougle CJ, Naylor ST, Cohen DJ, Aghajanian GK, Heninger GR, Price LH. Effects of tryptophan depletion in drug-free adults with autistic disorder. Arch Gen Psychiatry 1996; 53: 993–1000.
63. Yang C-J, Tan H-P, Du Y-J. The developmental disruptions of serotonin signaling may involved in autism during early brain development. Neuroscience 2014; 267: 1–10.
64. Malkova NV, Yu CZ, Hsiao EY, Moore MJ, Patterson PH. Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. Brain Behav Immun 2012; 26: 607–16.
65. Attia E, Walsh BT. Anorexia nervosa. Am J Psychiatry 2007; 164: 1805–10; quiz 1922.
66. Dellava JE, Thornton LM, Lichtenstein P, Pedersen NL, Bulik CM. Impact of broadening definitions of anorexia nervosa on sample characteristics. J Psychiatr Res 2011; 45: 691–8.
67. Gillberg IC, Råstam M, Gillberg C. Anorexia nervosa 6 years after onset: Part I. Personality disorders. Compr Psychiatry 1995; 36: 61–9.
68. Baron-Cohen S, Jaffa T, Davies S, Auyeung B, Allison C, Wheelwright S. Do girls with anorexia nervosa have elevated autistic traits? Mol Autism 2013; 4: 24.
69. Anckarsäter H, Hofvander B, Billstedt E, Gillberg IC, Gillberg C, Wentz E, et al. The sociocommunicative deficit subgroup in anorexia nervosa: autism spectrum disorders and neurocognition in a community-based, longitudinal study. Psychol Med 2012; 42: 1957–67.
70. Tennoune N, Chan P, Breton J, Legrand R, Chabane YN, Akkermann K, et al. Bacterial ClpB heat-shock protein, an antigen-mimetic of the anorexigenic peptide α-MSH, at the origin of eating disorders. Transl Psychiatry 2014; 4: e458.
71. Sinno MH, Do Rego JC, Coëffier M, Bole-Feysot C, Ducrotté P, Gilbert D, et al. Regulation of feeding and anxiety by alpha-MSH reactive autoantibodies. Psychoneuroendocrinology 2009; 34: 140–9.
72. Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 2013; 31: 814–21.
73. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2013; 505: 559–63.
74. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature 2013; 504: 446–50.
75. Marí-Bauset S, Zazpe I, Mari-Sanchis A, Llopis-González A, Morales-Suárez-Varela M. Evidence of the gluten-free and casein-free diet in autism spectrum disorders: a systematic review. J Child Neurol 2014; 29: 1718–27.
76. Reichardt N, Duncan SH, Young P, Belenguer A, McWilliam Leitch C, Scott KP, et al. Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. ISME J 2014; 8: 1323–35.
77. MacFabe DF, Cain NE, Boon F, Ossenkopp K-P, Cain DP. Effects of the enteric bacterial metabolic product propionic acid on object-directed behavior, social behavior, cognition, and neuroinflammation in adolescent rats: relevance to autism spectrum disorder. Behav Brain Res 2011; 217: 47–54.
78. Hosseini E, Grootaert C, Verstraete W, Van de Wiele T. Propionate as a health-promoting microbial metabolite in the human gut. Nutr Rev 2011; 69: 245–58.
79. Vinolo MAR, Rodrigues HG, Hatanaka E, Sato FT, Sampaio SC, Curi R. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. J Nutr Biochem 2011; 22: 849–55.
80. Currenti SA. Understanding and determining the etiology of autism. Cell Mol Neurobiol 2010; 30: 161–71.
81. Ustianowski A, Shaffer R, Collin S, Wilkinson RJ, Davidson RN. Prevalence and associations of vitamin D deficiency in foreign-born persons with tuberculosis in London. J Infect 2005; 50: 432–7.
82. Maxwell SM, Salah SM, Bunn JEG. Dietary habits of the Somali population in Liverpool, with respect to foods containing calcium and vitamin D: a cause for concern? J Hum Nutr Diet Off J Br Diet Assoc 2006; 19: 125–7.
83. Antico A, Tozzoli R, Giavarina D, Tonutti E, Bizzaro N. Hypovitaminosis D as predisposing factor for atrophic type A gastritis: a case-control study and review of the literature on the interaction of Vitamin D with the immune system. Clin Rev Allergy Immunol 2012; 42: 355–64.

84. Smolders J, Schuurman KG, van Strien ME, Melief J, Hendrickx D, Hol EM, et al. Expression of vitamin D receptor and metabolizing enzymes in multiple sclerosis-affected brain tissue. J Neuropathol Exp Neurol 2013; 72: 91–105.
85. Joseph RW, Bayraktar UD, Kim TK, St John LS, Popat U, Khalili J, et al. Vitamin D receptor upregulation in alloreactive human T cells. Hum Immunol 2012; 73: 693–8.
86. Wu S, Liao AP, Xia Y, Li YC, Li J-D, Sartor RB, et al. Vitamin D receptor negatively regulates bacterial-stimulated NF-kappaB activity in intestine. Am J Pathol 2010; 177: 686–97.
87. Olliver M, Spelmink L, Hiew J, Meyer-Hoffert U, Henriques-Normark B, Bergman P. Immunomodulatory effects of vitamin D on innate and adaptive immune responses to Streptococcus pneumoniae. J Infect Dis 2013; 208: 1474–81.
88. Ruemmele FM, Garnier-Lengliné H. Transforming growth factor and intestinal inflammation: the role of nutrition. Nestlé Nutr Inst Workshop Ser 2013; 77: 91–8.
89. Sczesnak A, Segata N, Qin X, Gevers D, Petrosino JF, Huttenhower C, et al. The genome of th17 cell-inducing segmented filamentous bacteria reveals extensive auxotrophy and adaptations to the intestinal environment. Cell Host Microbe 2011; 10: 260–72.
90. Yang J, Rose DJ. Long-term dietary pattern of fecal donor correlates with butyrate production and markers of protein fermentation during in vitro fecal fermentation. Nutr Res 2014; 34: 749–59.
91. Freedman LS, Commins JM, Moler JE, Arab L, Baer DJ, Kipnis V, et al. Pooled results from 5 validation studies of dietary self-report instruments using recovery biomarkers for energy and protein intake. Am J Epidemiol 2014; 180: 172–88.