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REVIEW ARTICLE

Helicobacter pylori and gut microbiota in multiple sclerosis versus Alzheimer's disease: 10 pitfalls of microbiome studies

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Abstract

Alteration of microbiota has been associated with intestinal, inflammatory and neurological diseases. An abundance of "good bacteria," such as *Bifidobacterium*, or their products has been generally believed to be beneficial for any diseases, whereas "bad bacteria," such as pathogenic *Helicobacter pylori*, are assumed to be always detrimental for hosts. However, this is not the case when we compare and contrast the association of the gut microbiota with two neurological diseases, multiple sclerosis and Alzheimer's disease. After *H. pylori* infection, pro-inflammatory T helper (Th)1 and Th17 immune responses are initially induced to eradicate bacteria. However, *H. pylori* evades the host immune response by inducing Th2 cells and regulatory T cells that produce anti-inflammatory interleukin-10. Suppression of anti-bacterial Th1/Th17 cells by regulatory T cells might enhance gastric *H. pylori* propagation, followed by a cascade reaction involving vitamin B_{12} and folic acid malabsorption, plasma homocysteine elevation, and reactive oxygen species induction. This can damage the blood-brain barrier, leading to accumulation of amyloid-β in the brain, a hallmark of Alzheimer's disease. In contrast, this suppression of pro-inflammatory Th1/Th17 responses to *H. pylori* has protective effects on the hosts, as it prevents uncontrolled gastritis as well as suppresses the induction of encephalitogenic Th1/Th17 cells, which can mediate neuroinflammation in multiple sclerosis. The above scenario might explain why chronic *H. pylori* infection is positively associated with Alzheimer's disease, whereas it is negatively associated with multiple sclerosis. Finally, we list "10 pitfalls of microbiota studies," which will be useful for evaluating and designing clinical and experimental microbiota studies.

> "The wolf shall live with the lamb,
> the leopard shall lie down with the kid,
> the calf and the lion and the fatling together,
> and a little child shall lead them."
>
> — Isaiah 11.6[1]

Introduction

In the "Peaceable Kingdom" of the Bible, humans co-exist peacefully with carnivorous and herbivorous animals. Although it sounds unrealistic, in the human gut, archaea, bacteria, fungi, parasites and viruses live peacefully as members of commensal microbes, beyond the "kingdom," and even beyond the domain. In the classification of life, there are three domains: *Bacteria*, *Archaea* and *Eukarya* (Eukaryotes), whereas viruses are not included into any domains.[2] Each domain is subdivided into the following ranks: the phylum, class, order, family, genus and species. In the Bible's Peaceable Kingdom, humans belong to the domain *Eukarya*, kingdom *Animalia*, phylum *Chordata*, class *Mammalia*, order *Primates*, whereas all the other animals also belong to the class *Mammalia*. The wolf, leopard and lion belong to the order *Carnivora* (wolves, family *Canidae*; leopards and lions, family *Felidae*), and the lamb

(sheep) and calf (cattle) belong to the order *Artiodactyla*, family *Bovidae* (sheep, genus *Ovis*; cattle, genus *Bos*). In addition to mammals, the phylum *Chordata* includes vertebrata including fish and frogs, and non-vertebrata, such as sea urchins and sea anemones.

In humans, the gut microbiota consists of approximately 1000 species of bacteria, five genera of archaea, 66 genera of fungi and as yet undetermined families of viruses including bacteriophages.[3] Currently, most microbiota studies are focused on the community of bacteria (bacteriome), but not the other taxa.[4,5] Healthy gut bacteriome mainly consist of two major phyla, *Bacteroidetes* and *Firmicutes*, and three minor phyla, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* (Table 1).[6,7] The phylum *Actinobacteria* is a group of mostly Gram-positive bacteria, which consists of 222 genera, such as the genera *Actinomyces*, *Collinsella* and *Streptomyces*.[8] The phylum *Bacteroidetes* is a group of Gram-negative bacteria, which consists of 128 genera, such as the genera *Alistipes*, *Bacteroides* and *Prevotella*.[9] The phylum *Firmicutes* is a group of Gram-positive bacteria, which consists of 241 genera, including well-known pathogenic bacteria, such as the genera *Bacillus*, *Clostridium*, *Staphylococcus* and *Streptococcus*.[10] The phylum *Proteobacteria* is the largest group of Gram-negative bacteria, which consists of 452 genera that include a variety of pathogenic bacteria, such as the genera *Brucella*, *Escherichia*, *Helicobacter* and *Salmonella*.[11] The phylum *Verrucomicrobia* is a group of Gram-negative bacteria with wart-like prosthecae, which consists of 12 genera, including the genus *Akkermansia*.[12]

Since the introduction of next-generation sequencing, there has been a growing number of studies on the association between microbiota and diseases.[13] In the present review article, we first introduce how gut microbiota changes have been

Table 1 Classification of bacteria associated with multiple sclerosis and its animal models

Phylum	Class	Order	Family	Genus	Species
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Adlercreutzia, Collinsella	
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	Fragilis
			Porphyromonadaceae	Butyricimonas, Parabacteroides	
			Prevotellaceae	Alloprevotella	
				Prevotella	Copri
			Rikenellaceae	Alistipes	
			S24-7		
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	
	Clostridia	Clostridiales			Segmented filamentous bacteria
			Christensenellaceae		
			Clostridiaceae	Clostridium	Perfringens
			Eubacteriaceae	Eubacterium	
			Lachnospiraceae	Anaerostipes, Blautia	
			Ruminococcaceae	Anaerotruncus, Faecalibacterium	
Proteobacteria	α-proteobacteria	Rhizobiales	Brucellaceae	Mycoplana	
	β-proteobacteria	Burkholderiales	Oxalobacteraceae	Undibacterium	Oligocarboniphilum
	δ-proteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila, Desulfovibrio	
	ε-proteobacteria	Campylobacterales	Helicobacteraceae	Helicobacter	Pylori
	γ-proteobacteria	Enterobacteriales	Enterobacteriaceae		
		Pasteurellales	Pasteurellaceae	Haemophilus	
		Pseudomonadales	Pseudomonadaceae	Pseudomonas	
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Akkermansiaceae	Akkermansia	

Phyla *Actinobacteria* and *Firmicutes* are Gram-positive, whereas phyla *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia* are Gram-negative.

associated with a variety of diseases, and move on to discuss the role of gut microbiota in multiple sclerosis (MS), its related neuromyelitis optica (NMO) and animal models. Then, we will propose the contrasting roles of *Helicobacter pylori* infection between MS versus Alzheimer's disease (AD). Finally, we list "10 pitfalls of microbiota studies" for microbiota study design and evaluation.

Microbiota changes and diseases

"Dysbiosis," an altered state of the bacterial community, has been associated with health conditions and diseases. While antibiotics are used to treat bacterial infections in humans and animals,[14] continuous use of broad-spectrum antibiotics can induce changes of the gut microbiota.[15] Antibiotics treatment decreases native bacterial species and disrupts the bacterial interactions, which potentially leads to the growth of harmful species, such as *Clostridium difficile*,[16–18] resulting in antibiotic-associated diarrhea.[16] "Probiotics" containing *Lactobacillus* species, such as *L. reuteri*, which naturally inhabits the mammalian gut, have been clinically tried to prevent antibiotic-associated diarrhea.[19]

The gut microbiota has been shown to play a crucial role in the induction of several immune components, such as T helper (Th)17[20] and MAIT cells.[21,22] Thus, changes in the gut microbiota have been associated with inflammatory diseases, particularly in the gastrointestinal tract (Table 2).[23] Inflammatory bowel diseases (IBD) have been considered to reflect interactions between microbes and the host;[24] changes in the gut microbiota have been reported in both ulcerative colitis and Crohn's disease.[25,26] Early childhood exposure to antibiotics is associated with an increased risk for Crohn's disease in which microbial diversity is diminished.[27,28] Necrotizing enterocolitis is another disease associated with the alteration of gut microbiota, although the precise pathogenesis is unclear. Necrotizing enterocolitis is primarily seen in premature infants,[29,30] whose clinical signs include feeding intolerance, increased gastric residuals, abdominal distension, and bloody stools.[31] Microbiome studies showed increased relative abundance of the phylum *Proteobacteria*, and decreased phyla *Firmicutes* and *Bacteroidetes*.[32] *L. reuteri* supplementation might reduce the risk of necrotizing enterocolitis.[33]

Changes of the gut microbiota have also been suggested to affect distant anatomical sites. Representative extraintestinal diseases, which have been associated with the gut microbiota, are listed in Table 2, including liver diseases,[34–37] atopic diseases,[38] diabetes mellitus (DM),[39–41] rheumatoid arthritis,[42] MS and AD.

Gut microbiota in MS and its animal models

Gut microbiota in MS

Multiple sclerosis is an inflammatory demyelinating disease of the central nervous system (CNS).[43] Although the precise pathomechanism is unclear, autoimmunity, genetic background and environmental factors, such as infections and latitude, appear to contribute to disease onset and exacerbation.[44] Among environmental factors, the gut microbiota has also been proposed to be associated with the pathogenesis of MS.[13,45,46] In high-income countries, the Westernization of lifestyle, including food, water and sanitation, has decreased several infectious diseases, such as viral hepatitis and helminth infestations, whereas chronic inflammatory and autoimmune diseases, including MS and IBD, have increased.[47] In particular, the "Western diet," which is rich in fat and salt, has been associated with the

Table 2 Diseases associated with gut microbiota

Disease	Microbiota association	References
Antibiotic-associated diarrhea	*Clostridium difficile* (pF)↑ *Lactobacillus reuteri* (pF) therapy	16,23
Inflammatory bowel disease: ulcerative colitis and Crohn's disease	Patient-specific fecal microbiota changes Species: *Escherichia coli* (pP)↑, *Proteus vulgaris* (pP)↑, *Enterobacter cowanii* (pP)↑, *Serratia marcescens* (pP)↑, *Candida tropicalis* (kF)↑	25–27,138
Necrotizing enterocolitis	*Lactobacillus reuteri* (pF) therapy Phylum: *Proteobacteria*↑, *Firmicutes*↓, *Bacteroidetes*↓	29,30,33
Extra-intestinal diseases	Liver diseases, atopic diseases, diabetes mellitus, rheumatoid arthritis, multiple sclerosis, and/or Alzheimer's disease may be influenced by antibiotics treatment, microbiota changes, or *Helicobacter pylori* (pP) infection	34–42

↑, increased relative abundance in patients; ↓, decreased relative abundance in patients; kF, kingdom *Fungi*; pF, phylum *Firmicutes*; pP, phylum *Proteobacteria*.

increased incidence of MS and IBD.[48,49] Fatty acids, as well as sodium chloride (NaCl), have been shown to increase Th17 cells, decrease regulatory T cells (Tregs) and exacerbate an animal model for MS.

Changes of the gut microbiota have been investigated in MS, by sequencing 16S ribosomal (r) RNA that is encoded in bacteria and archaea, but in neither fungi nor viruses.[45] Case-control studies showed that the microbiome of MS patients differs from that of controls, although it is unknown whether the altered microbiota is a cause or result of the development of MS (Table 3). In MS, reproducible changes of microbial taxa are limited, partly because each study often analyzed microbiome at different taxonomic ranks. For example, some studies showed the changes at the phylum and genus levels, whereas most studies showed the data neither at the order nor class level; the data in each study sometimes are incomparable. At the phylum level, Miyake et al. reported a decreased abundance of the phyla *Firmicutes* (e.g. genera *Faecalibacterium* and *Anaerostipes*) and *Bacteroidetes* (e.g. genus *Prevotella*) in the fecal microbiome of relapsing-remitting (RR)-MS patients, compared with healthy controls (HC).[50] Inconsistent with Miyake's findings, Chen et al. reported decreased phyla *Bacteroidetes* (e.g. genera *Parabacteroides* and *Prevotella*) and *Actinobacteria* (e.g. genera *Adlercreutzia* and *Collinsella*), as well as increased phyla *Firmicutes* (e.g. genus *Blautia*) and *Proteobacteria* (e.g. genera *Pseudomonas*, *Mycoplana* and *Haemophilus*) in RR-MS patients, compared with HC.[51] On the other hand, in pediatric RR-MS patients, Tremlett et al. reported an increase in the phylum *Actinobacteria*, but not in the other phyla.[52]

At the genus level, Jangi et al. reported a significant increase of the genus *Akkermansia* (phylum *Verrucomicrobia*) and a decrease of the genus *Butyricimonas* (phylum *Bacteroidetes*) in RR-MS patients.[53] At the species level, Rumah et al. reported unexpectedly that *Clostridium perfringens* type A (phylum *Firmicutes*) was present in 23% of MS patients, compared with 53% of healthy controls, whereas *C. perfringens* type B (natural host, ruminant animals) was detected in one MS patient.[54] As *C. perfringens* type A can cause food poisoning and gas gangrene, the reduction of such a potential pathogenic bacterium in MS patients is intriguing.

Table 3 Microbiota in multiple sclerosis and its animal models

Demyelinating diseases	References
MS	
Phylum level: *Actinobacteria* (pA)↑↓, *Bacteroidetes* (pB)↓, *Firmicutes* (pF)↑↓, *Proteobacteria* (pP)↑↓, *Verrucomicrobia* (pV)↑	50–54
Family level: *Coriobacteriaceae* (pA)↓, *Bacteroidaceae* (pB)↓, *S24-7* (pB)↓, *Christensenellaceae* (pF)↑, *Lachnospiraceae* (pF)↓, *Ruminococcaceae* (pF)↓, *Desulfovibrionaceae* (pP)↑, *Enterobacteriaceae* (pP)↑, *Helicobacteraceae* (pP)↓, *Akkermansiaceae* (pV)↑	
Genus level: *Adlercreutzia*(pA)↓, *Collinsella* (pA)↓, *Butyricimonas* (pB)↓, *Parabacteroides* (pB)↓, *Prevotella* (pB)↓, *Blautia* (pF)↑, *Haemophilis* (pP)↑, *Helicobacter* (pP)↓, *Mycoplana* (pP)↑, *Pseudomonas* (pP)↑, *Akkermansia* (pV)↑	
Species level: *Clostridium perfringens* (pF)↓, *Helicobacter pylori* (pP)↓	
EAE	
Members of the phyla *Firmicutes* (pF) and *Proteobacteria* (pP) were increased in rats, but not in mice	67,68
High fat diet reduced members of families *Prevotellaceae* (pB) and *S24-7* (pB)	68
SPF mice developed more severe EAE than GF mice	62,66
Oral antibiotic treatment suppressed EAE	64
SPF MOG-TCR Tg mice developed spontaneous EAE, while GF MOG-TCR Tg mice did not develop EAE	66
Polysaccharide A derived from *Bacteroides fragilis* (pB) suppressed EAE	63
TMEV-IDD	
Phylum level: *Bacteroidetes* (pB)↑↓, *Firmicutes* (pF)↑↓	82
Family level: *Rikenellaceae* (pB)↑, *Eubacteriaceae* (pF)↑, *Streptococcaceae* (pF)↓	
Genus level: *Alistipes* (pB)↑, *Eubacterium* (pF)↑, *Streptococcus* (pF)↓	
Oral antibiotic treatment did not influence demyelination	82

↑, increased relative abundance in patients/animal model; ↓, decreased relative abundance in patients/animal model; EAE, experimental autoimmune encephalomyelitis; GF, germ-free; MOG-TCR Tg, myelin oligodendrocyte glycoprotein-specific T-cell receptor transgenic; MS, multiple sclerosis; pA, phylum *Actinobacteria*; pB, phylum *Bacteroidetes*; pF, phylum *Firmicutes*; pP, phylum *Proteobacteria*; pV, phylum *Verrucomicrobia*; SPF, specific pathogen-free; TMEV-IDD, Theiler's murine encephalomyelitis virus-induced demyelinating disease.

Gut microbiota in NMO

Zamvil's group has proposed that the gut microbiota is also associated with NMO.[55,56] They found that T cells from NMO patients responded to aquaporin (AQP)4 peptide, p63-76, greater than those from HC. As p63-76 contains "10 residues with 90% homology" to a sequence p204-217 within *C. perfringens* adenosine triphosphate-binding cassette (ABC) transporter permease (TP), the authors suggested a potential pathogenic role of *Clostridium* species in NMO. Here, it should be noted that the "90% homology" is between the AQP4 p66-75 and ABC-TP p207-217, neither of which is a T-cell epitope,

but only a portion of the epitope. As (i) the real homology between AQP4 p63-76 and ABC-TP p204-217 is just 64% (9/14); and (ii) there is no evidence that *C. perfringens* infection induces T-cell responses to ABC-TP, it is unlikely that the immune response to *C. perfringens* could lead to generation of cross-reactive responses to AQP4. In addition, although the authors reported a robust proliferative T-cell response to p61-80 in all 15 NMO patients, Matsuya et al. found that only one in 12 NMO patients had the p61-80-specific T-cell proliferative response.[57] More recently, Zamvil's group analyzed the gut microbiome in NMO, compared with HC and MS samples, and detected 42 operational taxonomic units, which were differentially detected only between NMO versus HC, not MS versus HC.[58] Among 42 operational taxonomic units, *Enterobacteriaceae* of unknown species (4.08-fold) and *Prevotella copri* (0.11-fold) were the most and least abundant compared with HC, whereas *C. perfringens* was only 1.12-fold more abundant than HC (its *P*-value was the second least, though).

Gut microbiota in experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune model for MS. EAE can be induced by sensitization with myelin components, such as myelin oligodendrocyte glycoprotein (MOG) and myelin proteolipid protein.[59] The presence of the gut microbiota has been shown to affect EAE induction. In wild-type C57BL/6 mice sensitized with MOG, germ-free mice showed less severe EAE than specific pathogen-free mice, whereas germ-free mice transplanted with Th17-cell-inducing segmented filamentous bacteria (phylum *Firmicutes*, order *Clostridiales*, strong similarity with the genus *Clostridium*)[60,61] were more susceptible to EAE than control germ-free mice.[62] Ochoa-Rapáraz et al. showed that polysaccharide A derived from *Bacteroides fragilis* suppressed EAE.[63] Oral antibiotics administration in C57BL/6 mice before EAE induction reduced the clinical signs by enhancing interleukin (IL)-10 production from B cells.[64] In transgenic SJL/J mice expressing MOG-specific T-cell receptor on $CD4^+$ T cells,[65] gut commensal microbiota is required for induction of spontaneous EAE, although germ-free wild-type SJL/J mice sensitized with MOG showed only delayed onset compared with specific pathogen-free wild-type mice.[66]

Stanisavljević et al. reported that some members of the phylum *Firmicutes* and *Undibacterium oligocarboniphilum* (phylum *Proteobacteria*) were increased in the feces of rats with EAE.[67] In contrast, Haghikia et al.[68] showed that EAE itself did not alter the microbiome, whereas a high-fat diet exacerbated EAE with a reduction of the families *Prevotellaceae* and *S24-7* (proposed family name is "*Candidatus Homeothermaceae*"[69]) of the phylum *Bacteroidetes* in the feces of C57BL/6 mice. Although the precise pathophysiology of how fatty acids together with microbiota could contribute to CNS inflammation in EAE is unclear, these results are intriguing, as (i) some fatty acids are generated in the gut as fermentation products of dietary fibers by commensal bacteria;[70,71] and (ii) MS-like CNS inflammation is induced in X-linked adrenoleukodystrophy, whose principal biochemical alteration is the accumulation of very long-chain fatty acids.[72]

Gut microbiota, viral infections and a viral model for MS

In viral infections, the gut microbiota has been shown to promote viral replication. Kuss et al. showed that the intestinal microbiota can promote enteric replication of poliovirus (order *Picornavirales*, family *Picornaviridae*, genus *Enterovirus*), as orally antibiotic-treated mice had lower susceptibility to poliovirus-induced disease with decreased viral replication.[73] As poliovirus can bind certain bacterial lipopolysaccharides and peptidoglycans, the interactions might enhance the infectivity of poliovirus, although the exact pathomechanism remains unclear. Kane et al. showed that lipopolysaccharide from the gut microbiota was a key factor for successful transmission of mouse mammary tumor virus (family *Retroviridae*, genus *Betaretrovirus*) from the mother to offspring mice, as antibiotics treatment of the mother prevented the viral transmission.[74] The lipopolysaccharide-bound mouse mammary tumor virus activated dendritic cells (DC) and macrophages through Toll-like receptor 4, which induced IL-10. The production of IL-10 might inhibit anti-viral immune responses, resulting in the successful viral transmission. Jones et al. showed that histo-blood group antigens derived from enteric bacteria were required for effective norovirus infection in B cells. In an *in vitro* infection model of human norovirus (family *Caliciviridae*, genus *Norovirus*), viral replication in B cells was higher in the presence of histo-blood group antigens than in the absence of histo-blood group antigens by enhancing the attachment to B cells.[75] Furthermore, in an *in vivo* model of mouse norovirus, antibiotic-treated mice had

significantly lower viral titers in the intestine compared with the controls.

Theiler's murine encephalomyelitis virus (TMEV; family *Picornaviridae*, genus *Cardiovirus*) has been used to induce a viral model for MS.[76–80] As TMEV is a natural enteric pathogen in mice, the virus can infect the intestine.[81] Carrillo-Salinas et al. monitored the changes in the gut microbiome in TMEV-infected SJL/J mice, where relative abundances of bacteria differed significantly compared with uninfected control mice at the phylum and genus levels.[82] The oral administration of antibiotics of broad-spectrum depleted the gut microbiota and enhanced viral replication in the CNS with 50% mortality (TMEV infection alone did not kill any mice), whereas no clinical or histological effects were observed during the chronic phase. As the numbers of $CD4^+$ and $CD8^+$ T cells decreased in the cervical and mesenteric lymph nodes and the CNS, the depletion of the gut microbiota seemed to suppress anti-viral immunity, resulting in fatal acute viral infection, although anti-virus specific immune responses were not investigated in that study.

H. pylori infection in MS and AD

H. pylori infection in gastric and extragastric diseases

In the above section, it is intriguing that the presence of a potential pathogenic *C. perfringens* type A in feces was lower in MS than in HC. A similar negative association between a pathogen and MS has been reported in *H. pylori* infection. *H. pylori* is a spiral-shaped, flagellated, highly motile Gram-negative bacterium that selectively colonizes the human stomach (Fig. 1).[83,84] *H. pylori* belongs to the phylum *Proteobacteria*, class *Epsilonproteobacteria*, order *Campylobacterales*, family *Helicobacteraceae*, genus *Helicobacter*. *H. pylori* infects approximately 50% of the world's population, and its persistent infection in the gastric mucosa is etiologically associated with peptic ulcer, chronic gastritis, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma.[85–87] The standard therapy for eradication of *H. pylori* is treatment with a proton pump inhibitor and antibiotics, such as amoxicillin, clarithromycin and metronidazole.[87,88] *H. pylori* eradication has been shown to reduce the incidence of gastric cancer.[89,90] Mice and Mongolian gerbils are often used for *H. pylori* infection studies.[91–93] Although *H. pylori* Sydney strain 1 chronically infects mice and induces antibodies against *H. pylori*, infected mice showed only mild clinical signs and inflammation without development of gastric cancer. In contrast, *H. pylori*-infected Mongolian gerbils had severe inflammation in the stomach and developed gastric cancer.[92] Mouse and gerbil models reflect asymptomatic infection and symptomatic gastritis in humans, respectively.

H. pylori infection has also been associated with extragastric diseases. Idiopathic thrombocytopenic purpura (ITP) is a well-known disease associated with *H. pylori* infection;[94] *H. pylori* eradication results in a significant increase in the platelet counts in ITP patients. Although a link is not as strong as that of

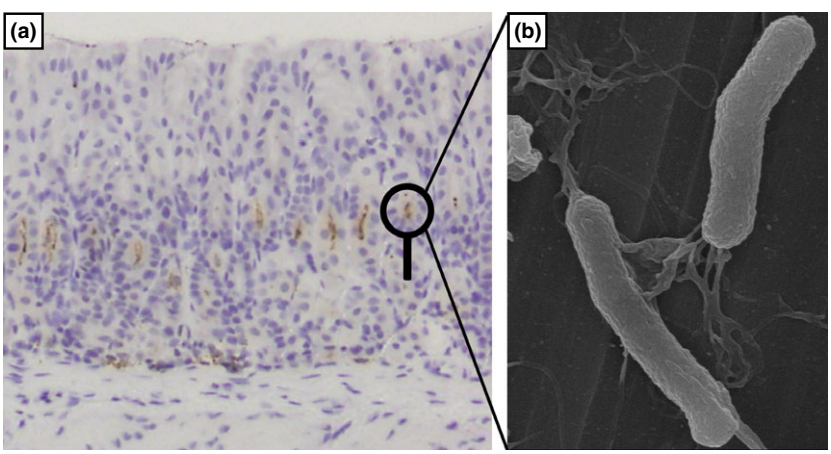

Figure 1 A chronically *Helicobacter pylori*-infected mouse stomach and electron microscopic image of *H. pylori*. (a) Light micrograph of *H. pylori* detected by immunohistochemistry (Thermo Fisher Scientific, Fremont, CA, USA; brown dots) in the stomach of a CD1 mouse, 6 months after *H. pylori* inoculation. Note that inflammation and tissue damage were not detected despite the presence of *H. pylori*. (b) Electron micrograph of *H. pylori* with the spiral shape and multiple flagella. Image was taken by an ultra-high resolution scanning electron microscope S-900 (Hitachi, Ibaraki, Japan) at Kindai University Faculty of Medicine (Osaka, Japan).

ITP, *H. pylori* infection has also been associated with cardiovascular diseases (CVD), immune-mediated diseases and neurological diseases. Similar to ITP, *H. pylori* infection appears to increase the risk of CVD,[95] DM[96] and AD.[97–99] In contrast, *H. pylori* infection seems to decrease the risk of asthma,[100–102] IBD[103] and MS.[104–106] Thus, *H. pylori* infection might play contrasting roles in two neurological diseases: a detrimental role for AD and a protective role for MS.

H. pylori infection is a protective factor against MS

Since 2007, Kira's group has shown that *H. pylori* seropositivity rates are lower in MS than in controls in Japan.[44,107,108] In the UK, the seroprevalence of *H. pylori* was half in MS patients compared with that of HC,[106] whereas Pedrini et al.[105] found that *H. pylori* seropositivity was lower in female patients, but not in male patients, compared with controls, using 550 Caucasian MS serum samples in Australia. Jaruvongvanich et al. carried out a meta-analysis of six observational studies from Japan, China, Iran, Greece, India and Australia, involving 1902 participants.[104] They showed a significant lower prevalence of *H. pylori* infection in MS patients. In contrast, in NMO, *H. pylori* seropositivity rates were significantly higher than controls.[109] Furthermore, *H. pylori* seropositivity was significantly higher in AQP4 antibody-positive patients than in AQP4 antibody-negative patients.[110]

Experimentally, Cook et al. tested whether infection of live *H. pylori* could affect EAE.[106] C57BL/6 mice were infected orally with 1×10^9 colony-forming units of the Sydney strain 1 of *H. pylori* every third day and sensitized with MOG for EAE induction, 3 weeks after *H. pylori* infection. *H. pylori*-infected mice showed lower clinical signs with decreased levels of MOG-specific lymphoproliferation, and reduced frequencies of Th1 and Th17 cells in the CNS and spleen, compared with the controls. Thus, *H. pylori* infection could ameliorate EAE by regulating immune responses to MOG. In addition, flow cytometric analyses of spleen $CD4^+$ cells showed decreased frequencies of Th1 and Th17 cells, as well as interferon-γ and IL-17 producing cells (after PMA/ionomycin incubation[111]) in infected mice, suggesting that general immune responses were also changed by *H. pylori* infection. Boziki et al. examined the effects of inactivated *H. pylori* in EAE. C57BL/6 mice developed EAE by the standard approach, sensitization with MOG emulsified in incomplete Freund's adjuvant containing inactivated *Mycobacterium tuberculosis* (known as complete Freund's adjuvant).[112] In contrast, sensitization with MOG emulsified in incomplete Freund's adjuvant containing inactivated *H. pylori* failed to induce EAE in C57BL/6 mice.[91] Thus, inactivated *H. pylori* might not have adjuvant effects.

Although these results are consistent with clinical seroprevalence of *H. pylori* in MS, the experimental setting might not reproduce *H. pylori* infection in humans. Generally, human *H. pylori* infection is established in childhood by 4 years-of-age, whereas the environmental factors during early life have been proposed to affect MS susceptibility.[113,114] In Cook's study, mice were sensitized with MOG just 3 weeks after *H. pylori* infection.[106] At this early phase of *H. pylori* infection, pro-inflammatory Th1 responses have been shown to function as a major effector cell.[115] During the chronic phase, anti-inflammatory Treg/Th2 responses become predominant with production of IL-10. It will be intriguing to test how chronic *H. pylori* infection affects EAE; for example, by transferring encephalitogenic T cells into chronically infected mice (passive EAE), which might provide clinically relevant information about the association between *H. pylori* infection and MS.

H. pylori infection is associated with AD progression

Alzheimer's disease is a progressive neurodegenerative disorder that is the most common form of dementia. The two histological features that define AD are neurofibrillary tangles and extracellular β-amyloid peptide (Aβ) deposits within senile plaques in the CNS. Unlike MS, *H. pylori* infection has been positively associated with AD. A significantly high prevalence of *H. pylori* infection in AD patients has been reported in Europe and East Asia, except Japan.[97–99,116]

To evaluate the effect of *H. pylori* eradication on the progression of AD, Chang et al. analyzed the data of patients who were diagnosed with AD and peptic ulcer with ($n = 675$) or without ($n = 863$) *H. pylori* eradication, in which AD patients received triple or quadruple therapy with proton pump inhibitors or H_2 receptor blocker, antibiotics (clarithromycin, metronidazole, amoxicillin or tetracycline) or with bismuth ($_{83}$Bi). Compared with no *H. pylori* eradication, *H. pylori* eradication was associated with a decreased risk of AD progression. Interestingly, in that study, there were significantly lower comorbidities of CVD and DM in AD patients with *H. pylori* eradication than those with no *H. pylori* eradication. Although no animal research has been carried out to investigate the association between

H. pylori infection and AD, mouse models of AD will be useful to clarify the role of *H. pylori* infection in AD.[117]

Blood-brain barrier (BBB) breakdown is one of the characteristics of neuroimaging and neuropathology of MS, which can be visualized by gadolinium enhancement magnetic resonance imaging or albumin and immunoglobulin (Ig) immunostaining of brain sections. Although such substantial BBB breakdown is not seen in AD, dysfunction of BBB has been shown in AD and its animal models.[118] As BBB restricts the transport of peptides from the periphery to the brain, BBB dysfunction can lead to accumulation of peripheral Aβ in the brain and/or decreased clearance of brain Aβ. We hypothesize the mechanism by which *H. pylori* infection leads to dysfunction of BBB (Fig. 2). First, chronic *H. pylori* infection increases pH in the stomach as a result of the parietal cell loss caused by atrophic gastritis and intestinal metaplasia.[119–121] The pH change decreases the absorption of vitamin B_{12} and folic acid, which increases homocysteine in the blood. Homocysteine is a metabolic intermediate of methionine, whereas vitamin B_{12} and folic acid metabolite (N^5-methyltetrahydrofolate) function as coenzymes when homocysteine is recycled into methionine or converted into cysteine. Thus, the deficiencies of vitamin B_{12} and folic acid increase the blood homocysteine level. Auto-oxidation of homocysteine generates hydrogen peroxide, which damages vascular endothelial cells, a component of BBB;[122] homocysteine-induced endothelial toxicity has been shown in isolated aorta and endothelia cells.[123] Then, subsequent BBB dysfunction and blood flow decrease caused by the high homocysteine level in the blood result in increased Aβ accumulation.[124] The high serum homocysteine level has been proposed to be a risk factor of AD and vascular diseases.[125,126] In addition, as described above, *H. pylori* infection is associated with increased comorbidities of CVD and DM, both of which can also cause BBB dysfunction.[118]

Distinct roles of *H. pylori* in MS versus AD

Although the gut microbiome has not been investigated in AD, some infections with bacteria, including spirochetes (*Borrelia burgdorferi* and *Treponema pallidum*[127,128]) and *Chlamydophila pneumoniae*,[129,130] have been associated with AD. Furthermore, *Chlamydophila pneumoniae* detection in brain tissues using a polymerase chain reaction method showed that 74% of AD patients were positive, whereas that of controls was 11%.[130] Using an AD model, $APP_{S\text{-}WE/PS1\Delta E9}$ mice, Minter et al. showed reduced amyloid plaque deposition with significant changes in the gut microbiome by long-term treatment with a cocktail of eight antibiotics for the duration of the 6-month lifespan.[131] They suggested that gut microbiota diversity might impact Aβ deposition.

Although the beneficial effect of antibiotics treatment on the murine AD model is similar to that on EAE models, why is the effect of *H. pylori* infection on MS and AD the opposite (Table 4)? There are two possible factors contributing to the distinct roles of *H. pylori* infection in the two neurological diseases. First, in *H. pylori* infection, higher gastric inflammation correlates with lower bacterial loads. *H. pylori* can control both innate and acquired

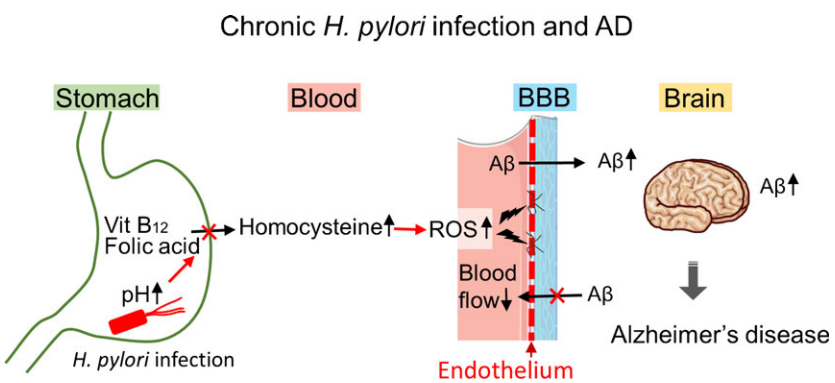

Figure 2 *Helicobacter pylori* infection might contribute to progression of Alzheimer's disease (AD). Chronic *H. pylori* infection has been known to increase gastric pH. This pH change decreases absorption of vitamin B_{12} and folic acid absorption, while it increases the blood homocysteine level. Auto-oxidation of homocysteine generates reactive oxygen species (ROS), which damages vascular endothelial cells, leading to blood–brain barrier (BBB) dysfunction and blood flow reduction. BBB dysfunction can not only increase the accumulation of amyloid-β (Aβ) from the periphery, but can also decrease the clearance of Aβ from the brain, contributing to progression of AD.

Table 4 Microbial and immune responses of multiple sclerosis and Alzheimer's disease

	Multiple sclerosis	Alzheimer's disease
Microbiota dysbiosis	+	?
H. pylori infection	Protection	Disease progression
T cell infiltration	+++	–
Immune response	Th1/Th17	Innate
Vascular/BBB dysfunction	+++	++

BBB, blood–brain barrier; *H. pylori, Helicobacter pylori*; Th, T helper.

immune responses in the hosts. *H. pylori* has been shown to activate, manipulate and evade pathogen recognition receptors, such as Toll-like receptors and C-type lectin receptors, on DC. If *H. pylori* activates pro-inflammatory genes and cytokines through pathogen recognition receptors on DC, the DC induce anti-bacterial Th1/Th17 responses that contribute to the eradication of *H. pylori*.[132,133] However, uncontrolled Th1/Th17 responses could induce immune-mediated gastritis (immunopathology).[86] In contrast, if *H. pylori* induces anti-inflammatory genes and cytokines by manipulating pathogen recognition receptor pathways in DC, the DC induce anti-bacterial Treg responses with anti-inflammatory cytokine IL-10 production. IL-10 suppresses anti-bacterial Th1/Th17 responses, facilitating *H. pylori* persistence. However, this immunosuppression is protective for hosts, as it prevents gastritis. Thus, Tregs act as a double-edged sword in *H. pylori* infection (Fig. 3).[79]

Second, although both MS and AD have often been described as CNS diseases with "neuroinflammation,"[134] substantial perivascular T-cell infiltration is seen in MS, but not in AD. While microglia and astrocytes (resident innate cells) are activated in both MS and AD, pro-inflammatory peripheral cellular immune responses, particularly Th1 and Th17 cells, contribute to the pathogenesis only in MS. Thus, increased Treg/Th2 response in individuals with persistent *H. pylori* infection can suppress encephalitogenic Th1/Th17, protecting from MS. In contrast, although Th2 cells help antibody production, anti-*H. pylori* antibody has no role in eliminating this bacterium; suppression of cellular Th1/Th17 immunity leads to propagation of *H. pylori*, which subsequently leads to BBB dysfunction and AD progression as discussed above. Similarly, *H. pylori*-induced exacerbation of NMO can be explained by enhancement of humoral immunity; that is, enhanced production of anti-AQP4 antibody.

Are the effects of *H. pylori* infection in MS and AD accompanied with changes of the gut microbiota? It is controversial whether *H. pylori* infection affects the gut microbiota. Although a higher gastric pH of infected humans can increase the number and diversity of gastric microbiota,[135] *H. pylori* infection has been reported to cause no or little effect on gastric microbiota in most human or animal studies.[136] Recently, however, Kienesberger et al. reported that gastric *H. pylori* infection altered the gastric and intestinal microbiota in mice.[137]

"10 pitfalls of microbiota studies"

Gut microbiota studies have a number of potential pitfalls. In the last section of the present review, we have listed the potential "10 pitfalls of microbiota studies," which will be helpful in evaluating and planning the microbiota study (Table 5).

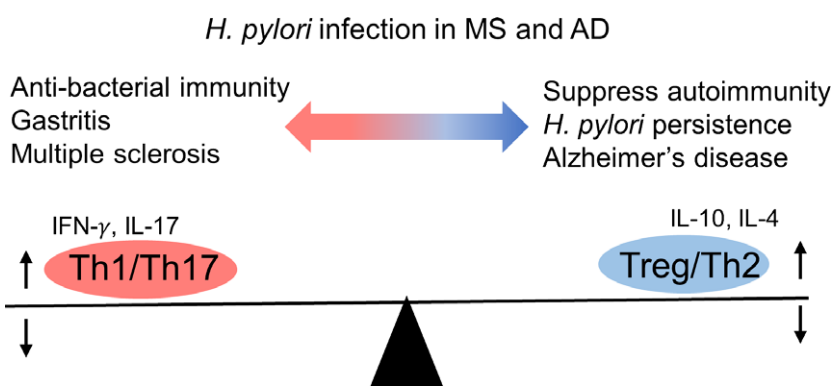

Figure 3 *Helicobacter pylori* infection in multiple sclerosis (MS) and Alzheimer's disease (AD). Chronic *H. pylori* infection changes the T helper (Th) cell subset balance toward regulatory T cells (Treg)/Th2 responses, suppressing MS and gastritis, both of which are mediated by pro-inflammatory Th1/Th17 responses. In contrast, increased Treg/Th2 responses can suppress anti-bacterial Th1/Th17 immunity, which leads to persistent *H. pylori* infection, resulting in BBB dysfunction and AD progression (see Fig. 2).

Table 5 Ten pitfalls in microbiota studies

Pitfalls	References
1. The term "microbiota" does not include fungi or viruses	138,139,142
2. Inappropriate usage of microbial taxonomy/ classification	143,144
3. The ratio of fecal bacterial taxa underrepresents the gut microbiota	145–147
4. Microbiota changes can be the cause or outcome of disease	3
5. Discrepancy between microbiota studies versus PID	148–152
6. Microbiota influenced by age, gender, and country	3,138,153,154
7. Probiotics/prebiotics are not always beneficial for hosts	156,157
8. Effect of antibiotics treatment on systemic microbiota and immune system	21,82,158,160–163,165
9. FMT methodology and safety	167–170
10. Tailor-made gut microbiota therapy	171–173

FMT, fecal microbiome transplantation; PID, primary immunodeficiency diseases; Treg, regulatory T cells.

1. The term "microbiota" does not include fungi, viruses and parasites

Although the term "microbiota" should include bacteria, archaea, fungi, viruses, protozoa and helminths, the majority of the microbiota studies focused on the bacterial community (bacteriome), sequencing conserved 16S rRNAs, but not on the fungal (mycobiome) or viral (virome) community. Bacteriome and mycobiome studies showed the significant intra-kingdom and inter-kingdom microbial correlations.[138] In addition, the association between MS and *Candida* species (kingdom *Fungi*) has been reported.[139] The virome represents the viral component of the microbiome, which includes viruses infecting not only the hosts, but also bacteria (bacteriophages). Currently, the virome analyses still require establishment of a standard pipeline for the sample preparation and sequence analyses, as: (i) the factors involving the sample preparation,[140] such as centrifugation, temperature and filtration, for viruses are different from those for bacteria; for example, the standard sample preparation protocols for the gut bacteriome are good to harvest bacteriophages localized in bacterial cell bodies, but bad for cell-free virions;[141] (ii) viruses lack universally conserved genomic regions; and (iii) a single reference viral genome database containing all eukaryotic DNA/RNA viruses and bacteriophage is not available for identification of viruses in the virome.[142]

2. Inappropriate use of microbial taxonomy/ classification

In most gut microbiota studies, it is obvious that some researchers do not pay attention to the bacterial taxonomy or classification system. As we discussed in the Introduction, there are taxonomic ranks classifying the bacteria in the following order; phylum, class, order, family, genus and species. However, it is not unusual even in the top journal articles, discussing the phylum and genus levels indiscriminately; for example, in a sentence, "We found an increase in the phylum *Firmicutes*, which supports the theory of importance of bacteria belonging to the clostridial group" (here, the authors do not care which taxonomic rank their "clostridial group" means), "clostridial group" can be the class *Clostridia*, the order *Clostridiales*, the family *Clostridiaceae* or the genus *Clostridium*. While phylogenetic distances among the different kingdoms are not the same, the above sentence is as obscure as the following sentence: "We found an increase of the phylum *Chordata*, which supports the theory of importance of sea anemones and/or of herbivorous animals." The inappropriate bacterial taxonomic description is partly due to the changing bacterial taxonomy system; the changes at the phylum levels are not uncommon, while no consensus information about bacterial classification is readily available.[143,144] For example, Collins et al. classified the genus *Clostridium* into 14 clusters in 1994, and discussed the "need of major revision," as some clusters including IV and XIVa consisted of phenotypically heterogeneous bacteria. Thus, the *Clostridium* cluster system does not provide precise information about the bacterial classification, yet it is still widely used. This is in contrast to the viral taxonomy, updated regularly by the International Committee on Taxonomy of Viruses, whose reports are available online for free (https://talk.ictvonline.org/taxonomy/).

3. The ratio of fecal bacterial taxa underrepresents the gut microbiota

In most human studies, stool samples have been used to investigate the gut microbiome. The fecal microbiome, however, might not reflect the gut microbiome,[145] as the bacteriome of the digestive tract differs in each portion. Steams et al.[145] showed that the bacterial communities of colon biopsy samples were distinct from those in stool samples. In addition, most microbiome studies showed only the ratio of bacterial taxa in the contents of organs, such

as saliva and stool, but did not quantify the total numbers of bacteria, which require information about many parameters, such as the total volume of the contents, weight, water content and intestinal transit time.[146] Although microbiome associated with mucosa of the organs are more biologically significant than those in the contents of the organs,[147] mucosal microbiome analysis requires biopsy of the mucosal samples, which is not feasible in many human studies.

4. Microbiota changes can be the cause or outcome of disease

The changes in microbiota can be the cause or outcome of disease.[3] This is true even in digestive diseases whose connection with gut microbiota appears to be straightforward.[3] For example, dysbiosis might only reflect constipation or diarrhea, which changes the gut and colonic transit, and fecal output.

5. Discrepancy between experimental microbiota studies versus clinical primary immunodeficiency diseases

In microbiota research, despite the key defense role of innate immune components including neutrophils and macrophages, acquired immune components, particularly IgA, Th17 cells and Tregs, have been studied more extensively in experimental mice, which have been proposed to influence the gut microbiota and their related diseases.[148] Clinically, however, reports of primary immunodeficiency diseases sometimes do not support the roles of such immune components.[149] For example, gain-of-function mutations in the STAT1 result in imbalanced STAT signaling, reducing Th17 cells.[150] The patients with the gain-of-function STAT1 mutation are characterized by susceptibility to oral and esophageal *Candida* infections, whereas candidiasis rarely appeared in other parts of the gastrointestinal tract; neither bacterial infections nor diarrhea are characteristic among the patients.

IgA deficiency is the most common primary immunodeficiency disease;[151] for example, the incidence in the Arabian peninsula and Spain is 1:143 and 1:163, respectively. Although some individuals with IgA deficiency are susceptible to infectious and immune-mediated diseases or have altered *Escherichia coli* strain phylogenetic group distribution, most people with IgA deficiency are asymptomatic and healthy.[152]

6. Microbiota is influenced by many factors, including age, sex and country

Gut microbiota has been shown to be influenced by many factors, such as the genetic background of the hosts, diet, age, sex and country.[3,138,153] Among the taxonomic ranks, from the phylum to the genus, there is no consensus on the bacterial components of "healthy gut microbiota" or its alteration (dysbiosis) that can be applicable to all individuals, whereas it might be possible to find the stable bacterial components/amounts at the genus or species level. Hoarau et al. compared the gut microbiome between: (i) patients with Crohn's disease; (ii) their healthy family members; and (iii) unrelated healthy individuals living in the same area.[138] They showed that the difference in microbiome between (i) and (ii) was smaller than the difference between (ii) and (iii). Although sex difference does not seem to affect microbiome in general, there are some reports that disease susceptibility might be associated with the differences in gut microbiota between male and female mice.[154]

7. Probiotics/prebiotics are not always beneficial for hosts

Some groups of bacteria, *Bifidobacterium* and *Lactobacillus*, are regarded as "good bacteria," whereas other groups of bacteria, including *Clostridium*, are regarded as "bad bacteria." "Good bacteria," known as "probiotics," as well as "prebiotics" that favor propagation of "good bacteria," including a high-fiber diet and breast-feeding, seem to be beneficial for any conditions from gastrointestinal diseases to neurological diseases, whereas bad bacteria and dysbiosis are always bad for any conditions. This is not necessary the case. As we reviewed the above, *H. pylori* and *C. perfringens* infections might protect from MS. Infant botulism is acute, flaccid paralysis caused by *Clostridium botulinum*; notably, the infant is the only family member who is ill with a broad peak from 2 to 4 months-of-age, despite the fact that normal human infant microbiota contains mainly *Bifidobacterium* and *Bacteroides* species.[155] In addition, identified risk factors for infant botulism include breast-feeding and the ingestion of honey.[156]

In the prebiotics field, the modern Western diet has been linked to recent increases of the prevalence of many diseases. Aging has been shown to reduce diversity of the gut microbiota with increased bad bacteria (opportunistic species and pathobionts) and reduced good bacteria producing short-chain fatty

acids. Although this change is usually considered "dysbiosis," one might suggest that this could be an adaptation to the aged condition.[157] Currently, we do not know whether the "Western diet" is good or bad for senescence; at least, the average life expectancy is higher in industrialized Western countries than developing countries where people are eating more prebiotics.

8. Effect of antibiotics treatment on systemic microbiota and the immune system

To investigate the role of microbiota, microbiota is depleted by antibiotics, experimentally. In many mouse studies, antibiotics are provided through the most facile means available; for example, through the animal's water supply.[158] While oral administration of non-absorbable antibiotics, such as neomycin[147,159–161] and vancomycin,[21] can affect mainly the gut microbiota, some studies often use highly efficacious absorbable drugs, such as metronidazole[73–75] and trimethoprim/sulfamethoxazole,[154] for complete microbiota depletion (trimethoprim/sulfamethoxazole can deplete even some fungi).[82] Here, the systemic effects of the absorbable antibiotics should be taken into account, such as changes in other microbiota (e.g. altered microbiota in the nasal cavity might change CNS viral infection through the olfactory route, while lung inflammation has been shown to suppress EAE[162]) and immunomodulatory effects (e.g. trimethoprim/sulfamethoxazole can cause hematological and allergic adverse effects, while minocycline can suppress microglia[163]). In contrast, diet and dietary supplements, some of which are known as prebiotics, have also been shown to alter microbiota. For example, resveratrol, a natural polyphenol compound,[164] is known to have anti-oxidant and anti-inflammatory effects; more recently, resveratrol has been shown to suppress IBD models with alteration of the gut microbiota.[165] Thus, in some pathological conditions, the influence on microbiota needs to be considered, once such diet/dietary supplements are proved to have antibiotic/prebiotic functions.

9. Fecal microbiome transplantation methodology and safety

Experimentally, to assess whether the microbiota is responsible for disease phenotypes, fecal microbiome transplantation (FMT) has been used. One standard protocol is that FMT from the donor to recipient is carried out through oral gavage, whereas an alternate protocol is co-housing and/or litter swaps (also referred to as cross-fostering) of the two mouse strains, as mice are coprophagic; co-housing allows the microbiota of all the animals within the same cage to homogenize.[166] These protocols have the disadvantage that some microbes, such as fastidious anaerobic bacteria or enveloped viruses, could not survive the fecal preparation or in the gastric acid.

Clinically, FMT has been reported to be effective in several diseases, particularly *Clostridium difficile* infection.[167] Although the term "transplantation" sounds safe, FMT is, after all, to infect humans with large numbers and species of archaea, bacteria, fungi, and viruses whose components and pathogenicity are largely unknown. For example, recently, even archaea has been suggested to be a human pathogen,[168] while archaea had been believed to be non-pathogenic. Giant viruses, such as mimivirus, are a part of the gut microbiota. The potential pathology of giant viruses is unknown, while they are frequently missed by virome studies that use 0.22-μm filters.[169]

Wang et al. carried out a systematic review on a total of 1089 patients receiving FMT in 50 publications, and concluded that serious adverse events, including death and viral infections, are not rare.[170] When live or inactivated pathogens are given to humans; for example, as vaccine for infectious disease or helminth therapy in MS, adverse effects have been extensively investigated, even though such treatment usually involves only one known microbial species.[171] In addition, historical transmission of infectious diseases by medical procedures and human behavior, including blood transfusion, sexual intercourse, breast-feeding and kissing, has been extensively investigated. Thus, the safety of FMT should be thoroughly investigated more widely for future clinical application.

10. Tailor-made gut microbiota therapy

To avoid severe adverse effects of FMT and probiotics treatment, tailor-made treatment is required, when considering all the above points. For example, *L. reuteri* has been clinically tested in other gastrointestinal diseases in children, prophylactically[172] or therapeutically.[173,174] We are currently carrying out a randomized controlled trial to see whether *L. reuteri* DSM 17938 can be effective for pediatric chronic constipation. Targeting one microbe and/or its product in one specific disease condition among a defined age-group of recipients will be one safe approach to find the individualized therapeutic and

prophylactic intervention of human health and diseases associated with the gut microbiota.

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Conflict of interest

None declared.

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