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REVIEW

The role of gut microbiota in the gut-brain axis: current challenges and perspectives

Xiao Chen[1*], Roshan D'Souza[2*], Seong-Tshool Hong[1]✉

[1] BDRD Research Institute, JINIS Biopharmaceuticals Inc, 948-9 Dunsan, Bongdong, Wanju, Chonbuk 565-902, South Korea
[2] Department of Microbiology and Genetics and Institute for Medical Science, Chonbuk National University Medical School, Jeonju 561-712, South Korea
✉ Correspondence: seonghong@chonbuk.ac.kr
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ABSTRACT

Brain and the gastrointestinal (GI) tract are intimately connected to form a bidirectional neurohumoral communication system. The communication between gut and brain, knows as the gut-brain axis, is so well established that the functional status of gut is always related to the condition of brain. The researches on the gut-brain axis were traditionally focused on the psychological status affecting the function of the GI tract. However, recent evidences showed that gut microbiota communicates with the brain via the gut-brain axis to modulate brain development and behavioral phenotypes. These recent findings on the new role of gut microbiota in the gut-brain axis implicate that gut microbiota could associate with brain functions as well as neurological diseases via the gut-brain axis. To elucidate the role of gut microbiota in the gut-brain axis, precise identification of the composition of microbes constituting gut microbiota is an essential step. However, identification of microbes constituting gut microbiota has been the main technological challenge currently due to massive amount of intestinal microbes and the difficulties in culture of gut microbes. Current methods for identification of microbes constituting gut microbiota are dependent on omics analysis methods by using advanced high tech equipment. Here, we review the association of gut microbiota with the gut-brain axis, including the pros and cons of the current high throughput methods for identification of microbes constituting gut microbiota to elucidate the role of gut microbiota in the gut-brain axis.

KEYWORDS gut microbiota, the gut-brain axis, central nervous system, high throughput methods, next-generation sequencings

INTRODUCTION

The human intestine contains a massive and complex microbial community called gut microbiota. A typical human carries 100 trillion microbes in the body, referred to as microbiota, microflora, or normal flora (Zimmer, 2010). Particularly, the collection of intestinal microorganisms along the GI tract, gut microbiota, has been known to be essential to the health and well-being of the host. Thus, the composition of gut microbiota and its role on human health and disease became a booming area of research, presenting a new paradigm of opportunities for medical and food applications.

It has been known that the failure in functional interactions between host and gut microbiota results GI tract disorders such as inflammatory bowel disease (IBD), GI tract malignancies, cholelithiasis, etc (Eckburg et al., 2005). Recent studies further revealed that the altered compositions of gut microbiota in host are with more complicated diseases such as behavior disorders and metabolic disorders. Examples are various, including autism, hepatic encephalopathy, allergy, obesity, diabetes, atherosclerosis, etc. Statistical analysis on the variations in the composition of gut microbiota and following biological experiments proved that the relationship between gut microbiota and humans is not merely commensal, but rather a mutualistic relationship. It is now clear that gut microbiota contributes significantly to host physiology and phenotypes such as nutritional uptake and well-being, obesity, diabetes, metabolic syndrome, behavior, various neurological diseases, etc (Bercik et al., 2012).

Gut and brain originated from the same tissue, the neural crest, during embryogenesis tightly work in tandem, each influencing the other (Cattell et al., 2011). The communication between gut and brain is called the gut-brain axis. Recent evidences showed that gut microbiota plays a very important role

*These authors contributed equally to the work.

in the gut-brain axis to affect mental health. Also there are evidences that gut microbiota is associated with non-neurological conditions such as obesity, diabetes, metabolic syndrome via the gut-brain axis (Manco, 2012).

Despite of the significant role of gut microbiota in the gut-brain axis, the elucidation of the molecular mechanistic pathway on the role of gut microbiota in the gut-brain axis is not moving forward as expected. This is because the elucidation of the molecular mechanistic pathway of gut microbiota in the gut-brain axis depends on precise high throughput identification of the microbial organisms constituting gut microbiota. Although there were some techniques utilized to identify microbial organisms constituting gut microbiota, current technological advances make the high-throughput identification of individual microbial components of gut microbiota possible. Especially, the advent of next-generation sequencing (NGS) has enabled the metagenomic and meta-transcriptomic analysis for high throughput identification of microbial organisms constituting gut microbiota. In this review, we focused on the role of gut microbiota in the gut-brain axis as well as the current challenges confronted in the high throughput identification of microbial organisms constituting gut microbiota to elucidate the role of gut microbiota in the gut-brain axis.

COMPOSITION OF GUT MICROBIOTA AND ITS INTERACTIONS WITH HOST

The gut microbiota colonizes the GI tract immediately after birth and persists throughout the adult life. Mostly, the gut microbiota is composed of anaerobic bacteria, belong to either the *Bacteroidetes* or the *Firmicutes* phylum with minor proportions of other phyla such as *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria*, and *Cyanobacteria*. So far, multiple studies suggested various numbers of bacterial species, ranging from 1200 species up to 35,000 bacterial phylotypes in the human gut. It is generally accepted that, however, the human microbiota contains as many as 100 trillion bacteria, a number that is 10 times greater than the number of human cells, representing a combined microbial genome well in excess of the human genome (Bocci, 1992).

In terms of the function of host-microbe interaction, it makes sense to have outnumbered bacteria in our gut. The intestine harbors a diverse bacterial community that is separated from the internal environments by a single layer of epithelial cells. Host-microbe interaction occurs along mucosal surfaces in the intestine of host. By mutual interaction with host, gut microbiota play essential roles in providing vital functions to host, such as protection against epithelial cell injury (Rakoff et al., 2004), metabolic regulation (Stappenbeck et al., 2002), GI tract development (Stappenbeck et al., 2001), immunomodulation by development of innate and adaptive immune responses, and absorption of nutrients (Fredrik et al., 2005; Ben et al., 2008).

Considering the vast numbers of microbiota bacterial cells, it is surprising that the composition of the human microbiota is fairly stable and conserved at the phylum level. Within phyla, however, types and its frequencies of microbial organisms constituting gut microbiota in each individual are very different from each other at the species levels. The composition of the jut microbiota is established during the first few years of life and is likely shaped by multiple factors, including maternal vertical transmission, genetic makeup, diet, antibiotics, infections, and stress (Hopkins and Sharp, 2001; Lewis and Cochrane, 2007; Cecilia and Sonja, 2010; Brian et al., 2013). Since the jut microbiota is a key player of the mucosal homeostasis, dysregulation of the intestinal mucosa homeostasis is consequentially implicated in the progression of disorders. Extensive researches have been undertaken over the last decade in order to link the gut microbiota and its influence on the host physiology and phenotypes. Particular interesting topics with high potential for personalized management of gut microbiota are treatment of metabolic syndrome and related diseases, prevention and control of (recurrent) infections, immune mediated disorders, and the gut-brain axis (Wu et al., 2004; Cryan et al., 2011). The current genomic revolution offers an unprecedented opportunity to identify the molecular foundations of these relationships so that we can understand how they contribute to our normal physiology and how they can be exploited to develop new therapeutic strategies.

THE GUT-BRAIN AXIS

The ability of gut microbiota to communicate with the brain and thus modulate behavior is emerging as an exciting concept in health and disease. The brain-gut axis is a bidirectional communication system, comprised of neural pathways, such as the enteric nervous system (ENS), vagus, sympathetic and spinal nerves, and humoral pathways, which include cytokines, hormones, and neuropeptides as signaling molecules (Cryan et al., 2011) (Fig. 1). Since the ENS is involved in the control of merely all gut functions including motility, secretion, absorption, and blood flow, it is an essential component of the gut-brain axis strategically placed at the interface between the gut and the brain. When the gut microbiota composition is altered driving by medications, chemicals and so on, the intestinal epithelium cells could sense these changes and give signals to ENS by altering the hormone secretion. It is extremely receptive of chemical information arising from intestinal epithelium as well as the enteric endocrine and immune systems and provides input to sensory pathways that signal to brain areas involved in emotion and cognition (Cryan et al., 2011). On the other hand the ENS receives efferent information from the brain through autonomic neural connections (sympathetic and parasympathetic) and hormonal pathways, which in turn modulate digestive functions (Barbara et al., 2007).

In health, gut-brain interactions contribute to the regulation of digestive processes including GI motor function, secretion/absorption and blood flow as well as to the regulation of food intake, glucose metabolism, modulation of the gut-associated immune system and, synchronization of physical and emotional states impacting on the GI tract. In disease, however, altered

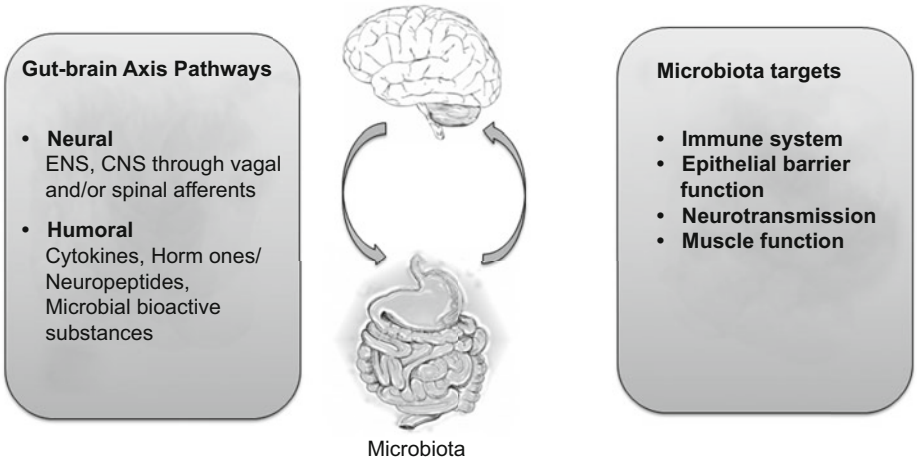

Figure 1. The gut-brain axis: Pathways of communication between brain and gut and microbiota targets. The gut microbiota always influences the host in neural and humoral manners which interplay and connect the brain and gut as an integral axis.

gut-brain signaling and interaction are likely to underlie the pathophysiology of various eating disorders, gastroesophageal reflux disease, nausea and vomiting, gastroparesis, functional GI disorders such as dyspepsia and irritable bowel syndrome (IBS). In addition, they might also contribute to systemic immune or mood disorders (Barbara et al., 2007). Therefore, the investigation of gut-brain axis could not only improve the understanding of GI disease, but also develop many potential medications for behavioral disorders and metabolic diseases, such as autism, obesity and diabetes.

The gut-brain axis and brain function

The gut-brain axis, a bidirectional neurohumoral communication system in human body, was well noted traditionally because of the correlated commodity between anxiety disorders and IBDs (Whitehead et al., 2002; Walker et al., 2008). However, it has not been understood how gut and brain communicate each other. Recent researches suggested that gut microbiota modulate the brain function of its host through the gut-brain axis (Turnbull et al., 1999; Finegold et al., 2002; Shanahan, 2002; Sudo et al., 2004; Desbonnet et al., 2008; Neufeld et al., 2011). The series of events in the GI tract following postnatal microbial colonization resulted a long-lasting impact on the neural processing of sensory information regarding the hypothalamic-pituitary-adrenal stress response (Finegold et al., 2002). Early postnatal bacterial colonization in germ-free (GF) mice promoted the development of central nervous system (CNS) (Turnbull et al., 1999). The c-Fos activation in the paraventricular nucleus was rapidly induced by the inoculation of *B. infantis* (Sudo et al., 2004). Further studies on the role of gut microbiota may clarify how and to what extent the neural and cytokine-mediated pathways can contribute to flora-mediated modulation of hypothalamic-pituitary–adrenal stress response. Also, *Clostridial* species were increased in the stools of children with autism than that in the stools of control (Neufeld et al., 2011). On the other hand, the mouse experiments confirmed that anxiety-like behavior and central neurochemical changes were relieved in GF mice compared with specific pathogen free (SPF) mice (Desbonnet et al., 2008). In addition, tryptophan metabolism was modulated by *B. infantis*, suggesting that the normal gut microbiota can influence the precursor pool for serotonin which is related to neurophysiological behavior (Shanahan, 2002). These studies strongly suggest that gut and brain communicate each other to modulate the brain function of its host through the gut-brain axis.

The gut-brain axis and behavioral phenotypes

Early life environmental influences have a profound impact on the organism's later development, structure, and function, such as the gut microbiota (Andrew, 2002). Immediately after birth, the newborn organism is rapidly and densely populated with complex forms of indigenous microbes. This process has been shown to contribute to developmental programming of epithelial barrier function, gut homeostasis, and angiogenesis, as well as the innate and host adaptive immune function, even the common neuro-developmental disorders, such as autism and schizophrenia, and microbial pathogen infections during the perinatal period (Andrew, 2002). The influence of gut microbiota persisted in the whole lifespan of organisms.

For gut-brain axis researches, the ideal model is the utilization of GF mouse, which are animals devoid of any bacterial contamination, offer the possibility to study the impact of the complete absence of a gut microbiota on behavior (Cryan et al., 2011). Sven Pettersson and co-workers carried out experiments on GF mice and SPF mice and found out the significant behavioral difference between them. Comparing with the SPF mice, GF mice showed the increased motor activity and reduced anxiety-like behavior, altered expression of synaptic plasticity-related genes, elevated noradrenaline (NA), dopamine (DA), and 5-hydroxytryptamine (5-HT) turnover in the

striatum, which proved their hypothesis that the "healthy" gut microbiota is an integral part of the external environmental signals that modulate brain development and function (Heijtz et al., 2011). However, once the gut microbiota community is interrupted by multiple factors including maternal vertical transmission, genetic makeup of the individual, diet, medications such as antibiotics, GI infections and stress, the gut homeostasis is subsequently imbalanced and causes detrimental effects on CNS through gut-brain axis (Heijtz et al., 2011), while some other experiments concluded that the gut microbiota influence brain chemistry and behavior independently of the autonomic nervous system, GI-specific neurotransmitters, or inflammation (Bercik et al., 2012).

On the other hand, the probiotics, which are beneficial in the treatment of the GI symptoms of disorders, had been clinically proved the role of probiotic intervention in reducing the anxiety and stress response as well as improving mood in IBS patients and those with chronic fatigue. The *Lactobacillus reuteri*, a potential probiotic known to modulate the immune system decreases anxiety as measured on the elevated plus maze as well as reducing the stress-induced increase of corticosterone. Although the mechanism of action is not known, some probiotics do have the potential to lower inflammatory cytokines, decrease oxidative stress and improve nutritional status (Cryan et al., 2011).

The gut-brain axis and metabolic disease

The metabolic diseases, such as obesity, diabetes and consequently atherosclerotic vascular disease have become major health and public health issues worldwide. With the long-time investigation of these increasingly epidemic metabolic diseases, the scientists discovered that only the individual nutritional habits are not enough to explain the high incidence, especially for obesity, they started to focus on the environmental factors that increase energy yield from diet, regulate peripheral metabolism and thereby cause body weight gain and insulin resistance. Extensive efforts of research revealed that the influence of gut microbiota is considered as a pivotal factor for development of these metabolic disorders. The regulatory peptide hormones, which are released from upper intestine and utilized by gut microbiota to communicate with the hypothalamus in brain (Bercik et al., 2011), are modulated though neural and endocrine pathways to control energy balance and glucose homeostasis, which is linked to obesity and diabetes in both human and mouse model (Migrenne et al., 2006; Sam et al., 2012).

Generally, gut microbiota synthesizes a large amount of glycoside hydrolases that break down complex plant polysaccharides to monosaccharides and short-chain fatty acids (SCFA), mainly acetate, propionate, and butyrate. In obese individuals, they harbor unique H_2-producing bacterial groups, particularly members of the *Prevotellaceae* family and certain groups within *Firmicutes* which facilitate the fermentation to produce much more SCFA (Greiner and Backhed, 2011). These SCFA are ligands for two G-protein-coupled receptors, Gpr41 and Gpr43, of gut enteroendocrine cells. Upon ligand binding, these G-protein-coupled receptors stimulate secretion of peptide YY (PYY), which inhibits gut motility and slows intestinal transit thereby enhancing nutrient absorption (Zhang et al., 2009) (Fig. 2). Besides the modulations of gut-derived peptide secretion, the induction of chronic low-grade endotoxinemia and regulation of tissular biologically active fatty acid composition are the other two mechanisms linking gut microbiota to obesity. All of these impacts on host can lead to the fat accumulation and body mass gain by gut-brain axis. Although the effective obesity therapeutics have been pursued for a long time, there are limited options for obese patients and the obesity pharmacotherapy always companied with severe side-effects, such as tachycardia and fluid derangements (Bercik et al., 2011). However, the application of gut hormones will modulate the gut composition to keep homeostasis instead of disrupting it and finally minimize metabolic deviations between diabetic patients and normalcy. Therefore, the gut hormones could also be a new treatment of obesity.

Diabetes is broadly considered as a metabolic disease resulting from genetic and environmental factors. Additionally, there is only less than 10% of the overall metabolic phenotype causing by point mutations and the low impact of genetics on metabolic diseases is further reinforced by the growing incidence of diabetes and obesity over the last decades. Since the obvious increase incidence of diabetes, especially type II diabetes, the genomic pathogen could not be the solely factor for its epidemicity. Subsequently, researches on gut microbiota of diabetic patients indicated that the proportions of phylum *Firmicutes* and class *Clostridia* were significantly reduced in the diabetic group compared to the control group. Furthermore, the ratios of *Bacteroidetes* to *Firmicutes* as well as the ratios of *Bacteroides-Prevotella* group to *C. coccoides-E. rectale* group correlated positively and significantly with plasma glucose concentration but not with body mass indexes (BMIs). Therefore, bacterial sequences, specific for type II diabetes rather than obesity, can be considered as signatures of hyperglycemic syndrome (Musso et al., 2010). Recently, scientists are interested in treatment of faecal microbiota transplantation to patients with bacterial infection after antibiotic therapy, although it remains a controversial treatment.

The gut-brain-liver axis

Glucose is the major energy source for many mammalian cells, and blood glucose levels are carefully maintained. The liver plays a major role in blood glucose homeostasis by maintaining a balance between the uptake and storage of glucose *via* glycogenesis and the release of glucose *via* glycogenolysis and gluconeogenesis. Maintaining blood glucose levels within a narrow range requires the regulation of two major metabolic pathways, gluconeogenesis and glycogenolysis, which produce glucose in the liver (Nordlie et al., 1999; Burcelin et al., 2011). Recently, the studies illustrated the relevance of a

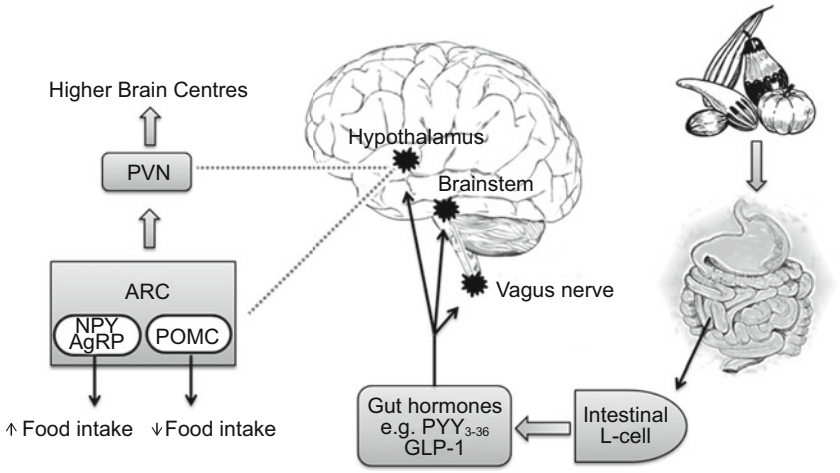

Figure 2. Regulation of food intake by gut-brain axis: Nutrients absorbed from intestine are proposed to activate G protein coupled receptors, e.g. the L-cell. The gut hormones are released after stimulations which may influence food intake at three sites: the vagus nerve, brainstem and hypothalamus. Two neuronal populations are considered as critical conduits which are related to food intake modulation, the orexigenic NPY/AgRP neurons and the anorexigenic POMC neurons. Further connections of higher brain centres may control the hedonic aspects of food ingestion. ARC (arcuate nucleus), AgRP (agouti related peptide), GLP-1 (glucagon like peptide-1), NPY (neuropeptide Y), POMC (propiomelanocortin), PVN (para-ventricular nucleus), PYY (peptide YY).

pathway where intestinal lipids regulate glucose homeostasis involving a gut-brain-liver axis (Fig. 3). Their findings indicated that the direct administration of lipids into the upper intestine increased upper intestinal long-chain fatty acyl-coenzyme A (LCFA-CoA) levels and suppressed glucose production even under the situation of sub diaphragmatic vagotomy or gut vagal deafferentation which interrupts the neural connection between the gut and the brain and blocks the ability of upper intestinal lipids to inhibit glucose production (Beraza, 2008). The discovery of a gut-brain-liver axis for regulating liver glucose homeostasis and the possible impact of insulin-resistance opens a wide variety of new therapeutic options to treat obesity and diabetes mellitus.

CURRENT HIGH THROUGHPUT METHODS FOR IDENTIFICATION OF MICROBES CONSTITUTING GUT MICROBIOTA

For elucidating the role of gut microbiota in the gut-brain axis, accurate identification of microbes constituting gut microbiota is the most important premise condition. Conventionally, culture-based surveys were employed to identify the human gut microbial diversity (Wang et al., 2008). However, only less than 1% of all microbial organisms in nature can be grown by culture-based approaches. Also, the culture media selection and incubation condition are time-consuming. Most importantly, the experimental results based on culture-based approaches do not reflect the population dynamics of actual microbial communities because it is not possible to culture whole set of microbial organisms without changing frequency of individual species in the microbial population. Therefore, rapid and accurate identification of individual microbial organisms in gut microbiota are urgently required so as to be able to elucidate the role of gut microbiota in the gut-brain axis. With recent technological developments especially in the area of NGS, it is now practically possible to identify all of microbial organisms constituting gut microbiota within several days.

Next generation sequencing approaches

New technologies for accurate and raid identification of bacteria are essential to epidemiological surveillance. For classifying and identifying bacterial species, cumbersome physiological, serological, biochemical, chemotaxonomic, and more recently genomic methods have been routinely applied in microbiology (Blaut et al., 2004). Polymerase chain reaction (PCR) based methods to detect microorganisms are available but cannot be used for classification, especially in the case of unknown bacterial samples (Sintchenko et al., 2007). DNA sequencing is one of the gold standards for the characterization of the bacteria, but this approach cannot be used for fast classification and identification. In general, these methods such as PCR require optimization for setting up specific assays for each bacterial strain.

To identify gut microbiome and push through the limitations encountered in the traditional culturing methods, the revolutionary method—NGS has been applied to achieve this target. Since the relative paucity of sequenced gene fragments and the use of fecal biota as substitute for the entire gut microbiota, that the diversity of micro-organisms is not well-defined could

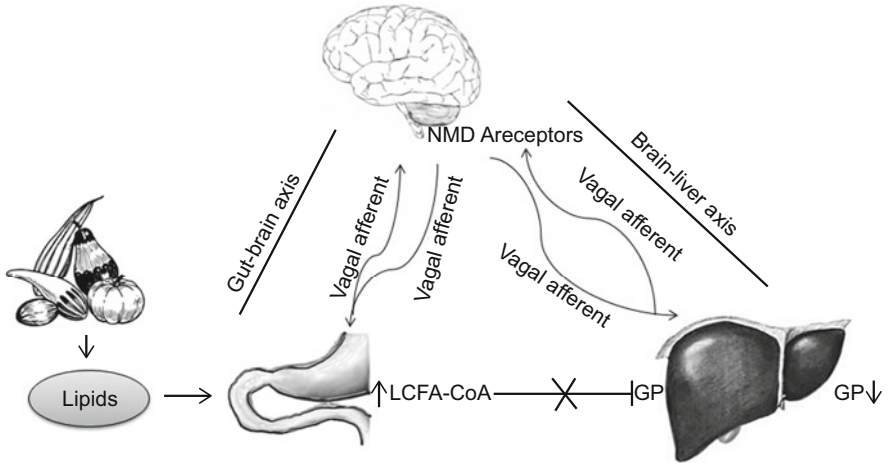

Figure 3. The gut-brain-liver axis: The glucose production in liver is modulated by upper intestine lipid absorption through gut-brain-liver axis, but not gut-liver direct interplay.

reduce the precision of this method. Considering that, Eckburg et al. undertook large scale comparative analysis using full length 16S rRNA sequences to clearly characterize the adherent mucosal and fecal microbial communities and to examine how these microbial communities differed between subjects and mucosal sites. Consequently, 62% of the 395 bacterial phylotypes were novel and, 80% represented sequences from species that have not been cultivated (Eckburg et al., 2005) and recently more than 15% sequences has been proved from new species (Zhang et al., 2011). In succession, many studies were performed based on the full length 16S rRNA to determine the extent of the bacterial diversity, 16S rRNA sequences are binned into operational taxonomic units (OTUs) according to sequence percentage sequence identity. OTUs containing sequences with >99% pairwise sequence similarity indicate "strain-level" taxa, while >97% designates "species", >95% ID "genus", and >90% ID "family" (Jock and Geider, 2004; Peterson et al., 2008). Thus, full length 16S rRNA sequencing strategy proves significant while assessing the microbial diversity in the gut microbiota.

Considering the cost in Sanger sequencing in microbial analysis, there have been several approaches through pyrosequencing. As a cost effective and time saving technique, this has been employed in several fields to identify the microbial communities in various atmospheres like deep sea and soil (Sogin et al., 2006). Pyrosequencing generates large number of 16S rRNA sequence tags by amplifying the select variable regions within the 16S rRNA, also achieving 100 folds higher throughput than Sanger sequencing. Better taxonomic resolution can be obtained due to targeting amplification of highly variable select regions of the gene i.e. V2, V3 or V6 regions of the 16S rRNA (Hamady, et al., 2008). Turnbaugh et al. have performed the studies on obese and lean twins (154 individuals in total) using the pyrosequencing technology and concluded that human gut microbiota is shared among family members, but that each person's gut microbial community varies in specific bacterial lineages present, with a comparable degree of co-variation between adult monozygotic and dizygotic twin pairs (Turnbaugh et al., 2008). Hence, Dethlefsen et al. have investigated the distal gut microbiota communities on humans before and after the treatment of antibiotics, obtaining 7000 full length rRNA sequences and over 900,000 pyrosequencing reads from two hypervariable sequence regions of the rRNA genes, by which they have identifies the 3300–5700 taxa from the samples they used (Dethlefsen et al., 2008). The latest developments of 454 and illumina technologies offer higher resolution and show relative consistency with each other (Claesson et al., 2010).

Recent studies have also focused upon the "metagenomic systems biology" approach, which can potentially advance metagenomic research in the same way systems biology advanced genomics, appreciating not only the part list of system but the complex interactions among parts and impact of these interactions on functions and dynamics such as the gut-brain axis. Making use of 454 FLX-derived data in addition to illumina-dervied shotgun metagenomic data helps in validating the results obtained. Annotating the metabolic sequence data to identify the enzymatic genes reveals the metabolic reactions thus helps in constructing the community-level metabolic network of the gut microbiota, which has linked bacterial community to the gut that is associated with the specific host phenotypes (Greenblum et al., 2012). Similarly, to study the gut microbiota community, Gill et al. have used whole genome shotgun sequencing and provided the relative species abundance by assembling the size and depth of the coverage of genome generated from metagenomics project (Gill et al., 2006). Fig. 4 illustrates the outline of the procedure being adopted in order to identify the individual bacteria using the NGS technology.

MALDI-TOF strategies

A further advance would be the application of matrix-associat-

ed laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), which provided high resolution proteomic-based comparisons of whole bacterial cells. Bacterial identification based on peptidic spectra obtained by MALDI-TOF mass spectrometry was proposed >30 years ago (Anhalt and Fenselau, 1975; Claydon et al., 1996; Krishnamurthy and Ross, 1996). It has only recently been used as a rapid, inexpensive, and accurate method for identifying bacteria. The taxonomic resolution of MALDI-TOF MS is currently considered as a comparable or superior method to comparative 16S rRNA gene sequence analysis. It is recognized that the MS of different bacterial species were distinct from one another, exhibiting the potential of becoming a species discriminating characteristic, analogous to genetic finger printing techniques such as amplified fragment length polymorphism (AFLP) or random amplification of polymorphic DNA (RAPD) (Holland et al., 1996; Welker, 2012). Use of this approach in conjunction with ongoing genomic data will greatly facilitate the recognition of gut microbiota composition. Strains giving rise to unidentified proteomic profiles may be subjected to gene sequence analysis to promote their detailed phylogenetic characterization. This will permit a parallel updating of proteomic and genomic databases which will provide an invaluable resource for future gut ecological studies. Fig. 4 shows the general scheme of MALDI-TOF MS based identification of micro-organisms, which compares the individual MS to the reference and rules out the specific species in the sample. MALDI-TOF MS allows the identification of various micro-organisms by so called intact-cell mass spectrometry and the comparison of a sample's mass spectrum to reference mass spectra in a database. The key factors to the success of this technology are the fact that a uniform sample preparation procedure is utilized for many different types of micro-organisms, comparatively low cost and short time per analysis (Angelakis et al., 2011). Additionally, MS-based identification can be readily expanded to different microbiological fields, including food, industrial and veterinary microbiology. Recently, MS has been implemented for bacterial identification from commercialized probiotic products. There has been discrepancy between the bacterial strain announced on the label on commercial product and strain identified using MALDI-TOF. MS presented 92% specificity compared to molecular assay and additional species were identified (Angelakis et al., 2011). Albesharat et al. have performed studies on microbial diversity using RAPD, which was further characterized by MALDI-TOF MS with better clustering result (Albesharat et al., 2011).

Genes expressed differentially between GF mice and SPF mice. GF mice displayed increased motor activity and reduced anxiety, compared with SPF mice with normal gut microbiota (Heijtz et al., 2011). However, there needs to be additional studies required in analyzing the direct role of the gut microbiota influencing the gut-brain axis. Animal-based research has extended the idea of microbiota-brain interactions to many psychiatric disorders and brain development. Studies indicate that behavioral changes induced by destabilization of the microbiota are likely to be mediated by substances of microbial origin acting on the host brain either directly or indirectly through neuro-active substances (Heijtz et al., 2011). However, it is still under debate, whether the gut microbiota metabolites are contributing towards the neural behavior or not. Traditionally, MALDI-TOF approach involved culturing the bacteria, followed by its spectrum analysis. Recent studies suggest that feces samples have been processed and directly fed to the instrument for analysis, by which the non-culturable bacteria can also be identified and help elucidating the gut-brain axis. Moreover, the MS approach presented allows the integration of data from different biological levels such as the genome and the proteome. It used protein mass pattern detection approach which is independent from DNA sequencing or PCR-based approaches (Collins et al., 2012). Lagier et al. analyzed 32,500 colonies by MALDI-TOF, found 340 different bacterial species among seven phyla and 117 genera. This included 174 species never described in the human gut (Lagier et al., 2012). However, most of these procedures have so far not exceeded proof-of-principle level and were applied only to a limited number of bacterial species (Holland et al., 1996; Chong et al., 1997). In addition, these procedures do not have proven maturity for easy and systematic application in microbiology. Consequently, biologists have not consistently utilized these approaches despite their great potential.

Recently, stable isotope probing approaches have been introduced, offering the great potential to identify microbes that are involved in the metabolism of specific substrates. Stable isotopes like ^{13}C or radioisotopes like ^{14}C are added to the samples and monitored for the phylogenetic information (Dumont et al., 2006). Raman microspectroscopy (Huang et al., 2007) and nano-secondary ion mass spectrometry (Kuypers and Jørgensen, 2007) also have been used which combine the single cell technologies with stable isotope analysis of microbial communities to monitor the single-cell level. In addition, Raman approach can be combined with fluorescence *in situ* hybridization (FISH) which facilitates the understanding the link between individual bacterial cells and their metabolic functions which will be of great significance in order to link the gut-brain axis (Dumont et al., 2006). However, this technology is far from becoming commonplace and affordable, because of the high cost and infrastructure required for the analysis.

Fluorescence *in situ* hybridization

The principle of FISH is the detection of a target DNA or RNA site by a fluorescently labeled probe molecule. The high sensitivity and specificity of FISH and the speed with which the assays can be performed have made FISH a powerful technique with numerous applications, and it has gained general acceptance as a clinical laboratory tool (Bishop, 2010).

Salminen and colleagues used this method to work on the gut microbiota composition in the first and third trimesters of pregnancy. They selected 36 normal pregnant women and 18

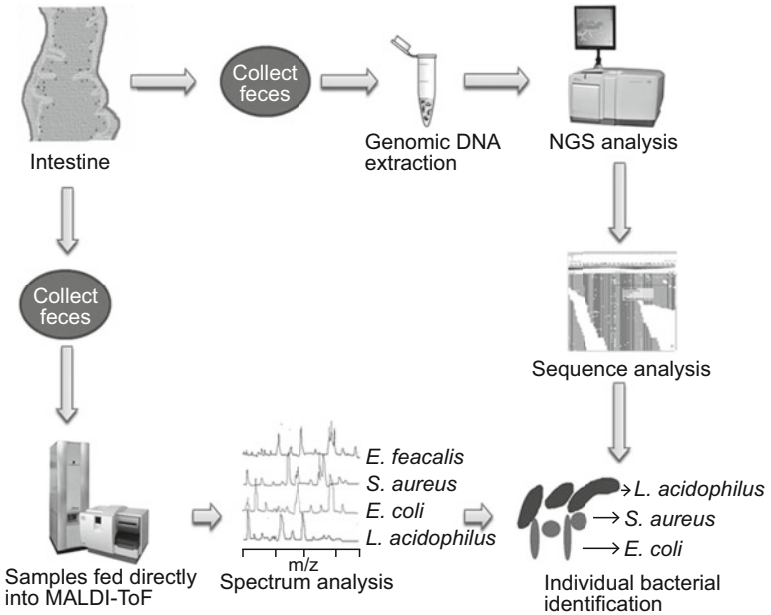

Figure 4. Schematic representation of the bacterial identification using NGS and MALDI-TOF platform: Genomic DNA is extracted from feces and analyzed using NGS and the sequence reads are compared with the database. Samples are directly fed into the MALDI-TOF, followed by its spectrum analysis to identify the bacteria present.

overweight pregnant women for experiment and found that the gut microbiota composition changed during pregnancy in normal-weight and overweight women according to weight gain over pregnancy (Collado et al. 2008). Certainly, the corrected probe design in these experiments is the guarantee of specific detection of gut microbiota (Kurz et al., 2011). As the analysis of gut microbiota, the knowledge of the 16S rRNA sequence information is imposed to design a probe that specifically targets a given organism. However, *in silico* designed probes may not work well in *in situ* hybridisation experiments which could reduce the accuracy of identification of gut microbiota.

DGGE/TGGE

Denaturing gradient gel electrophoresis (DGGE) works by applying a small sample of DNA (or RNA) to an electrophoresis gel that contains a denaturing agent. Researchers have found that certain denaturing gels are capable of inducing DNA to melt at various stages. As a result of this melting, the DNA spreads through the gel and can be analyzed for single components, even those as small as 200–700 base pairs. TGGE, a refinement of it, relies on temperature dependent changes in structure to separate nucleic acids. TGGE and DGGE can be applied to nucleic acids such as DNA and RNA, and (less commonly) proteins. They have been used to assess microbial community diversity in sludge, sub-seafloor biosphere, soil, biofilm, river, wastewater, crude oil, human intestines, insects, manure, probiotic products, cheese, milk, and fermented sausage (Wook et al., 2011). Recently, PCR-DGGE method is applied to detect bacterial partial 16S rRNA gene amplicons from ruminal fluid, aiming at the research of host feed efficiency under a low-energy diet for the first time. They found that PCR-DGGE bands represented specific bacteria to metabolites in the bovine rumen and linked with host feed efficiency traits, although the exact strain has not been figured out due to the limitations of the existing database (Hernandez et al., 2010).

Microarray

Microarray has been developed and validated for determining the microbiota diversity and evaluating the relative proportions of genus-like or higher (phylum-like) phylogenetic groups (Kim et al., 2005; Lotta et al., 2013). The first extensive DNA microarrays containing probes designed to detect members of the GI microbiota were developed in the Brown laboratory, which is based on an Agilent platform containing probes targeting up to 359 microbiota species, as well as up to 316 “novel OTUs” identified during studies of human colon and stomach microbial ecology (Palmer et al., 2006; Palmer et al., 2007). Microarrays represent an excellent choice for the high-throughput analysis of bacterial populations, because many different probes can be placed on one slide or chip, and samples thus can be tested for the presence of many different species simultaneously samples can be interrogated directly, circumventing any need for culturing, and thus non-culturable species can be reliably detected. It’s been demonstrated that microarray has a power equal to new generation sequencing (Claesson et al., 2009).

Several types of microarrays have been used to identify the gut microorganisms, like community genome array, functional genome array and phylogenetic oligonucleotide array (Loy et

al., 2002; Wu et al., 2004; He et al., 2007). Recently, Paliy et al. developed a more sensitive microarray containing Affymetrix GeneChip platform containing 775 representative sequences of phylospecies clusters obtained from human feces and several colon sites and was able to detect bacterial DNA present at the level of 0.00025% of the total community DNA (Paliy et al., 2009). The key factor in microarray is the stringency of the hybridization. Total DNA or RNA is isolated and fragmented, followed by PCR amplification and labeling. The labeled fragments are hybridized to oligonucleotide probes immobilized on a slide. This is visualized using a fluorescence scanner (Savage, 1977). A gut-specific phylogenetic microarray offers numerous benefits as it is a cost-effective and less time-consuming alternative to 16S sequencing methods, which provides similar levels of sensitivity, selectivity, and quantification.

CONCLUSION AND PROSPECT

The concept of gut microbiota as a determining factor for the various human phenotypes ranging from obesity to intelligence is one of the most noticed research topics in current sciences. Especially, the recent years are witnessing that gut microbiota regulate the human phenotypes through the gut-brain axis. Studies are revealing that gut microbiota seems to communicate with CNS through neural, endocrine and immune pathways to influence the physiology of body including the brain functions and behaviors of its host (Cryan and Dinan, 2012). However, clear elucidation of the role of gut microbiota in the gut-brain axis is confronted with a technological challenge of identifying microbes constituting the gut microbiota. The gut microbiota of a typical human is consisted of 100 trillion microbes, a number that is 10 times greater than the number of human cells (Zimmer, 2010). Although there are the pros and cons for current high throughput identification methods for identification of microbes constituting gut microbiota, NGS methods are the best approaches. After isolating whole genomic DNAs from a fecal sample, the high throughput identification of 16S rDNA by NGS alone generates enough information for high throughput identification of microbes constituting gut microbiota. Further computer-assisted statistical data analyses on the composition of gut microbiota, information of body phenotypes, and the status of the gut-brain axis will elucidate how gut microbiota affect and regulate the phenotypes of mammals through the gut-brain axis.

Finally, there is a possibility that the site-specific intestinal microbiota would affect the gut-brain axis. It has been well known that different microbes are enriched at the different sites of the GI tract (Simren et al., 2012). This result suggests that a different microbial composition at a specific location in the GI tract affect differently the phenotypes of host, because each locus of the GI tract not only functions differently but also uptake a different kind of nutrients (Sekirov et al., 2010). It would be difficult to answer whether the site-specific gut microbiota play a determinant role in the gut-brain axis. Intestinal microbe analyses on the fecal and tissue sample at the different sites of the GI tract obtained by using an enteroscope would answer the question on the role of site-specific intestinal microbiota in the gut-brain axis.

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ABBREVIATIONS

5-HT, 5-hydroxytryptamine; AFLP, amplified fragment length polymorphism; BMI, body mass index; CNS, central nervous system; DA, dopamine; DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence *in situ* hybridization; GF, germ-free; GI, gastrointestinal; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; LCFA-CoA, long-chain fatty acyl-coenzyme A; MALDI-TOF MS, matrix-associated laser desorption/ionization-time of flight mass spectrometry; NA, noradrenaline; NGS, Next-generation sequencing; OUT, operational taxonomic unit; RAPD, random amplification of polymorphic DNA; PYY, peptide YY; SCFA, short-chain fatty acid; SPF, specific pathogen free

COMPLIANCE WITH ETHICS GUIDELINES

Xiao Chen, Roshan D’Souza and Seong-Tshool Hong declare that they have no conflict of interest.

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