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Kertas Asli/*Original Articles*

Technology Advancement Enabling the Link of Gut Microbiota with Obesity and Metabolic Disorder

(Perkembangan Teknologi di dalam Menghubungkaitkan Mikrobiota Usus dengan Obesiti dan Gangguan Metabolik)

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ABSTRACT

Obesity is a growing epidemic due to an accelerated phase of industrialization and urbanization with the overfed people now outnumbered the underfed. It is the major public health problem with a lot of research interest as it is associated with many complicated chronic disorders such as type-2 diabetes, cardiovascular diseases (CVD) and cancers. A global estimation of 2.8 million deaths per year is due to obesity and there are tremendous on-going efforts to identify hosts and environmental factors that influence the cause and pathogenesis of obesity. Concerted efforts from different research groups had successfully shown that obese subjects have altered composition of gut microbiota and transplantation of this microbiota influences body weight in the germ-free recipient mice. The advancement of technology had made possible the study of gut microbiota which was unculturable for better understanding of their impact to human health. Rapid deep sequencing of DNA at reasonable cost through various options of platforms followed by data analysis using robust bioinformatic tools are an important way of analysing the gut microbiome. Here we review the role of gut microbiota which modulates host's metabolic functions and gene expression, facilitating the extraction and storage of energy from the ingested dietary substances and leading to body-weight gain. We will discuss on the different techniques used, focusing on the high-definition technologies for the determination of the composition, function and ecology of gut microbiota. This allows the appropriate selection of platform which becomes the key for success of subsequent research.

Keywords: Gut microbiota; obesity; metabolism; inflammations

ABSTRAK

Arus perindustrian dan pemodenan menjadikan obesiti sebagai suatu epidemik yang menular. Kini masyarakat yang mengamalkan pengambilan makanan yang berlebihan mengatasi mereka yang kekurangan makanan. Penyelidikan berkaitan obesiti kini menjadi tumpuan kajian memandangkan ia merupakan masalah utama kesihatan awam dan berkait rapat dengan penyakit kronik seperti kencing manis jenis 2, penyakit kardiovaskular dan kanser. Dianggarkan kematian yang berpunca dari masalah obesiti adalah sebanyak 2.8 juta orang di seluruh dunia dan pelbagai usaha sedang dilakukan bagi mengenal pasti faktor-faktor perumah dan persekitaran yang mempengaruhi punca dan patogenesis masalah ini. Usahasama dari beberapa penyelidikan telah membuktikan bahawa subjek yang obes mempunyai komposisi mikrobiota usus yang berbeza dan pemindahan mikrobiota mempengaruhi berat badan tikus penerima yang bebas kuman. Kemajuan teknologi membolehkan penyelidikan terhadap microbiota yang tidak boleh dikultur dan memberi pemahaman yang lebih mengenai impak organisma ini terhadap kesihatan manusia. Penjujukan DNA secara terperinci dan cepat dengan kos yang berpatutan melalui pelbagai platform disusuli penganalisaan data menggunakan bioinformatik yang moden adalah penting bagi menganalisa mikrobiom usus. Di sini kami meneliti peranan mikrobiota usus dalam mengawal atur fungsi metabolisma dan ekspresi gen perumah, membantu pengekstrakan dan penyimpanan tenaga dari makanan yang diambil dan seterusnya menyebabkan peningkatan berat badan. Pelbagai teknik berdefinisi tinggi turut dibincangkan di dalam pengenalpastian komposisi, fungsi dan ekologi mikrobiota usus. Ini akan dapat membantu dalam penggunaan teknologi yang bersesuaian untuk kejayaan penyelidikan.

Kata kunci: Mikrobiota usus; obesiti; metabolism; keradangan

INTRODUCTION

With the current state of acceleration in industrialization and urbanization, obesity has increased worldwide to the extent that those obese now outnumbered the malnourished people (Power & Schulkin 2008). WHO data indicates that currently, obesity affects at least 400 million people and it is the fifth leading risk for global deaths worldwide. About 65% of the world's population lives in countries where excess body weight kills more people than underweight

(WHO 2012). Obesity is not classified as a single disorder but currently characterized by a cluster of several metabolic disorders such as cardiovascular diseases, type-2 diabetes and cancers (WHO 2007; Sanz et al. 2010). Nevertheless, the exact pathogenesis of obesity and its related diseases are not well understood. Obesity is characterized by low grade, but persistent inflammation with increased production of cytokines and acute-phase reactants, such as C-reactive protein (CRP) which eventually leads to insulin resistance and metabolic syndrome (Wellen & Hotamisligil 2005). Although excessive intake of energy-dense foods and a sedentary lifestyle are often blamed for obesity epidemic, there are emerging evidences pointing that gut microbiota is also responsible for the gain of body weight (Shoelson et al. 2007; Musso et al. 2010). Recent researches have postulated that gut microbiota alter host energy metabolism leading to adiposity and weight gain (Creely et al. 2006; Cani et al. 2007; Samuel et al. 2008).

Until recently, our understanding on how gut microbiota affects metabolic diseases is limited. The specific bacteria populations and the altered metabolic pathways which trigger the development of pathological conditions are not well defined. The complexity of gut microbial ecology and its impact on health can be better understood by first knowing extensive coverage of microbial population in the gut. Without advanced technology, the data detailing microbial composition somehow lack comprehensiveness. Development of the non-culture-based analysis, such as metagenomics had revolutionised the advancement of medical microbiology in characterizing and identifying many clones which corresponds to novel species of microorganisms. Through metagenomics analysis, the full genome composition of microbiomes, and unique microbial genes associated with the microbiomes across the human body can be discovered. In addition, metabolomics approach can help expand our knowledge on the mechanisms that link gut microbiota to adipogenesis in both physiological and pathological condition of obesity (Turnbaugh et al. 2008).

In this review, we discuss the role of the gut microbiota in energy metabolism and inflammation; and their possible links with obesity and other metabolic disorders. We also describe the different techniques that are used to unravel the specific changes of the composition of gut microbiota which affect or counteract the development of metabolic disorders.

GUT MICROBIOTA

Gut microbiota is a complex community of trillion of bacteria dwelling the length and width of the mammalian gastrointestinal tract. The majority of these microbial reside in our gut, with density estimated between 10^{11} to 10^{12} cells / ml, and two kilograms heavier than our brain (MetaHIT 2010). This bulk of the community could be considered as our additional organ, with respect to their integration and contribution to the host's metabolism. Their genomes are also considered as 'other' genomes in the host, comprising more than 100 fold genes more than what human has.

More than 3 million bacterial genes have been reported in our gut alone, which varies and are unique to each of us (Weinstock 2012). Collectively, there are nine distinct phyla of microbiota such as Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria, Actinobacteria, Spirochaetes, VadinBE97 with Firmicutes and Bacteriodetes being the most dominant phyla residing in our gut ecosystem (Vrieze et al. 2010; Prakash et al. 2011; Dave et al. 2012). Firmicutes, a gram-positive group of bacteria contains more than 200 genera, including *Lactobacillus*, *Mycoplasma*, *Bacillus* and *Clostridium;* while the gram-negative Bacteriodetes have about 20 genera such *as Prevotella, Fusobacterium* and *Porphyromonas* (Vrieze et al. 2010).

Microhabitat variations throughout the gastrointestinal tract, such as pH, oxygen, and nutrient are some of the factors that influence specific types and compositions of gut bacteria. Based on conventional microbiological culture technique, anaerobic bacteria such as bacteroides, bifidobacterium and eubacterium are more prevalent at the lower portion of the gut, whereas the upper portion is mainly inhabited by aerobic bacteria such as escherichia, enterobacter, and enterococcus (Guarner & Malagelada 2003).

However, recent molecular based analysis showed that the same bacteria phyla, *Firmicutes* and *Bacteriodetes* are present at the different sites in the gut and that only the relative proportion of the subgroups of the common phyla varies. The subgroup of *Firmicutes*: the family *Streptococcaceae* was found dominant in small intestine while colon was enriched by *Bacteroidetes* phylum and *Lachnospiraceae* family of *Firmicutes* (Frank et al. 2007).

Each individual has distinct and highly diversified communities of gut microbes, although a similar set of gut colonizers which are the core gut microbiota are shared among individuals (Turnbaugh et al. 2009; Qin et al. 2010). Therefore, different microbial types which are present in different individuals need to be characterized as they play important roles in influencing the well-being of an individual, and perhaps the etiologies of some diseases.

MECHANISTICS INFLUENCE OF GUT MICROBIOTA ON ENERGY METABOLISM

The gut microbiota has been regarded as another 'important organ' that is involved in the regulation of energy homeostasis. The metabolic activities of the gut microbiota help the host to extract and store calories as fat, and part of the calories are extracted from luminal nutrients for microbial growth and proliferation. Studies on the relationship of the gut microbiota with obesity have uncovered the influence of gut microbiota composition on adiposity.

Bäckhed et al. (2004) showed that young, conventionally raised (CONV-R) C57BL/6 mice had 40%

higher body fat content and 47% higher epididymal fat content compared to germfree (GF) C57BL/6 mice, despite consuming less food. When they colonized GF mice with the intestinal microbiota of CONV-R mice, they found that these "conventionalized" animals experienced a 60% increase in body fat and epididymal fat within 2 weeks. The increase in body fat was accompanied by insulin resistance, adipocyte hypertrophy, and increased levels of circulating leptin and glucose. This occurred despite the conventionalized animals consumed less food compared to their germfree counterparts. In addition to the modulation of *de novo* lipogenesis, the investigators found that conventionalized mice had a higher uptake of monosaccharides from the gut into the portal blood. This could be partly described by the higher density of the small intestinal villi capillaries of conventionalized mice as compared to germ free counterparts. These findings support the hypothesis that the composition of the gut microbiota affects the amount of energy extracted from the diet as well as the culprits for many metabolic disorders.

Bäckhed et al. (2007) further their study to understand the mechanisms of resistance to diet-induced obesity in the germfree mice. Germfree or conventionalized mice were fed with a high-fat, high-carbohydrate western diet. In addition to the results obtained in 2004, similar energy content was observed in the stool of both groups of mice, indicating that mechanisms other than energy harvested in conventionalized mice may be responsible for the gain in fat mass. Finally, 2 complementary but independent mechanisms that result in the increased fatty acid metabolism in germfree mice which were resistant to diet-induced obesity have been proposed:

1. An increased activity of fasting-induced adipocyte factor (FIAF) activates the production of peroxisome proliferator-activated receptor coactivator, which is known to increase the expression of genes encoding regulators of mitochondrial fatty acid oxidation and;
2. An increased in the activity of adenosine monophosphate – activated protein kinase (AMPK), an enzyme that monitors the cellular energy status. AMPK will activate key enzymes of mitochondrial fatty acid oxidation, including acetyl-CoA carboxylase and carnitine palmitoyl transferase. These intriguing findings suggest that the gut microbiota has a suppressive effect on FIAF and AMPK activities, resulting in increased adiposity and insulin resistance in host.

In exploring the role of gut microbiota in host metabolism, the conventionalized mice were found to have an increased activity of hepatic carbohydrate response element-binding protein (ChREBP) and liver sterol response element-binding protein (SREBP-1) which promote fat deposition in the liver and increased insulin levels (Bäckhed et al. 2004). The gut microbiota therefore caused an increase of glucose uptake from the small intestine. This increase was associated with a high activity of glycosyl hydrolases in conventionalized mice, which are capable of digesting dietary polysaccharide. The glucose is subsequently converted into lipid in the liver. The lipogenic enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) are controlled by the 2 signaling proteins, ChREBP and liver SREBP-1 (Bäckhed et al. 2004; Denechaud et al. 2008). Interestingly, the conventionalization of FIAF-deficient knockout (KO) mice produced only a 10% extra total body fat compared to 60% fat gain observed in wild-type counterparts (Bäckhed et al. 2004).

A very recent evidence indicated that gut microbiota which produces *t10,c12* conjugated linoleic acid (CLA) enhances hepatic lipogenesis and triglyceride synthesis through mammalian target of rapamycin (mTOR) /SREBP1 pathway. In response to gut microbiota-producing t10,c12 CLA treatment, lipid accumulation occurs as a result of (1) enhanced incorporation of acetate, palmitate, oleate, and 2-deoxyglucose into triglycerides; (2) increased mRNA expression and protein levels of lipogenic genes, which include SREBP-1, acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FASN), elongation of very long chain fatty acids protein 6 (ELOVL6), glycerol-3-phosphate acyltransferase 1 (GPAT1), and diacylglycerol O-acyltransferase 1 (DGAT1) (Go et. al. 2013). Based on these evidences, extensive research is dedicated to differentiate the mechanisms that lead to lipogenesis and those that resulting in energy balance in host as potential therapeutic application (Parekh et. al. 2014).

Recent molecular studies have highlighted that the composition of intestinal microbiota in obese genetic models is different compared to lean wild-type animals. The obese animal model has mutations in the gene responsible for the production of leptin, a hormone that regulates energy intake and energy expenditure.

Ley et al. (2005) found that the obese genotype had 50% lower bacteroidetes prevalence and a significantly higher prevalence of firmicutes compared to lean wild-type mice. Using the same animal models, Turnbaugh et al. (2006) discovered that the gut microbiome of obese mice was enriched with sequences encoding for glycoside hydrolases.

Elevated concentrations of acetate and butyrate were also observed in the caecum of genetically obese mice compared to lean mice. The gut microbiota produces a large amount of glycoside hydrolases that break down complex polysaccharides from the diet into absorbable forms, i.e monosaccharides and short chain fatty acids (SCFAs). Acetate, propionate and butyrate are the main SCFAs produced by this fermentation process. SCFAs function as ligands which bind to the G-protein-coupled receptors (Gpr 41 and Gpr 43) of enteroendocrine cells.

Upon ligand binding, these receptors stimulate secretion of gut hormone peptide YY (PYY), which lead to reduced intestinal transit, increased energy harvest and stimulation of hepatic lipogenesis (Samuel et al. 2008). Turnbaugh et al. (2006) also reported that the metabolic characteristics associated with the obese-type microbiota include the increased adiposity, which was transmissible

through transplantation of the gut microbiota from *ob/ob* mice to germfree non-*ob/ob* mice. After being colonized with an obesity-type microbiota, adult C57BL/6J mice gained significantly higher body fat percentage and significantly higher caecal levels of firmicutes. *Bacteroides* species which are the key polysaccharide degraders are the most abundantly represented faecal microbiota.

There are 172 different genes found in *Bacteroides thetaiotaomicron* for polysaccharide utilization, compared with 39 genes encoded by *Bifidobacterium longum*. These genes allow *B. thetaiotaomicron* to metabolize a wide range of non-digestible plant polysaccharides into oligosaccharides and monosaccharides (Xu et al. 2003). *B. thetaiotaomicron* also appears to increase the activity of host monosaccharide transporters in the gut, promote angiogenesis and strengthen the mucosal barrier in germ free animals upon colonization, leading to increased body fat accumulation (Hooper et al. 2001; Stappenbeck et al. 2002; Hooper el al. 2003; Xu & Gordon, 2003).

Nevertheless, there are contradictory results on gut microbiota and adiposity. In the colonization studies of germ free animals, *B. thetaiotaomicron* which belong to the *Bacteriodete*s phylum was found to induce adiposity and body weight gain (Bäckhed et al. 2004; Faith et al. 2014). The same trend was observed in other studies which characterized by a higher levels of *Firmicutes* and a reduction in *Bacteriodetes* population (Ley et al. 2005; Turnbaugh et al. 2006). According to Tuohy et al. (2009), germfree animals may have gut microbiota that are different from the conventional animals. The gastrointestinal tract of conventionally reared animals develops and matures alongside its resident microbiota while germfree animals are in fact truly xenobiotic and do not exist in nature.

Their physiology and metabolism of the germ free mice were adapted for life without bacteria. The conventionalization of these animals with microorganisms would lead to a dramatic effect on their physiological processes. The conventionalized animals now have to cope with microbial derived metabolites, energy, antigens and signaling molecules. Thus the roles and mechanisms undertaken by the bacteroidetes in energy metabolism should be explored and understood.

The fact that *B. thetaiotaomicron* appears to induce obesity upon the colonization of germ free animals may be related to the unique single bacterium-germ free animal interactions which may be less important in conventional animals or in humans colonized by many hundreds of different bacterial species. *B. thetaiotaomicron* was shown to induce angiogenesis and vascularization of the intestine of germfree mouse, which greatly increased the ability of the host to absorb nutrients from the gut.

GUT MICROBIOTA, INFLAMMATION AND OBESITY

The disturbed gut microbiota rather than a single organism are often the pathologic agents of chronic diseases (Friedrich 2008), and this presumably means a different bacterial diversity and/or different degrees of overgrowth of the more aggressive residential bacteria, *i.e.*, bacteria which induce inflammatory responses via host's immune system (Hakansson & Molin 2011). Gut microbiota that are known to be pathogenic or opportunistically pathogenic in the healthy individuals are *Escherichia coli* (*E. coli*) and *Bacteroides fragilis* (*B. fragilis*). Increased proportions of *E. coli* and *B. fragilis* have also been linked to inflammatory bowel diseases (IBD) (Kleessen et al. 2002; Swidsinski et al. 2005; Wang et al. 2007).

Obesity and insulin resistance are associated with low-grade chronic systemic inflammation (Wellen & Hotamisligil 2005; Hotamisligil et al. 2006). Shi et al. (2006) found that gut microbiota initiate the inflammatory state of obesity and insulin resistance through the activity of lipopolysaccharide (LPS), which trigger inflammatory reactions by binding to the CD14 toll-like receptor-4 (TLR-4) complex at the surface of innate immune cells. LPS is a component of the gram-negative bacterial cell walls and very small amounts of LPS found in blood plasma of healthy human, ranging between1-200pg/ml, showing a healthy gut barrier (Moreira et al. 2012). Increased level of LPS in human is strongly associated with obesity and other metabolic disorders (Miller et al. 2009; Sun et al. 2010; Pussinen et al. 2011).

In an animal study, Cani et al. (2007) demonstrated that after 4 weeks of high-fat feeds, mice developed metabolic syndrome such as obesity, fasting hyperglycemia, steatosis, macrophages infiltration of adipose tissue, hepatic insulin resistance and hyperinsulinemia. The plasma LPS level increased progressively in these high-fat fed mice, and this condition is called metabolic endotoxemia. The investigators also found that dietary pattern changed the composition of gut microbiota with an increase ratio of gram-negative to gram-positive bacteria. The alteration of gut microbiota composition will lead to increased intestinal permeability through several mechanisms. The gut microbiota reduce the expression of host's genes coding for tight junction proteins ZO-1 and Occludin as well as increasing expression of anandamide and CB1 receptors (increased endocannabinoid system tone). The leaky gut will allows more LPS to enter the host circulation system, which triggers the activation of LPS receptor CD14, resulting in an increase of the inflammatory pathways. The LPS could enhance the number of preadipocytes (hyperplasia) which result in obesity (Luche et. al. 2013). Activin A secreted by macrophages isolated from obese adipose tissue plays an important role in proliferation and differentiation of human preadipocytes to adipocytes (Zaragosi et al. 2010).

In order to prove the causative link between LPS and metabolic diseases, CD14 mutant mice (CD14 knock-out mice – CD14KO) were fed with high-fat diet and/or a chronic low dose LPS infusion. CD14KO were completely resistant to the metabolic diseases caused by both, LPS and high-fat diet. In metabolic endotoxemia, CD14 activates the expression of inflammatory cytokines in adipose tissue such as tumor necrosis factor α (TNF-α), interleukin 1 (IL-1),

interleukin 6 (IL-6), and plasminogen activator inhibitor 1 (PAI-1) leading to metabolic disorders. CD14KO mice were found to be hypersensitive to insulin even when they were fed a normal diet and show delayed occurrence of obesity, diabetes and insulin resistance. Therefore, these findings support the hypothesis that the LPS/CD14 system sets the tone of insulin sensitivity and regulates the onset of obesity and diabetes (Cani et al. 2007).

In a subsequent study by Cani et al. (2008), antibiotic was used to treat the mice which were fed with high-fat diet and *ob/ob* mice. Antibiotic treatment altered the composition of the gut microbiota which reduced the cecal and plasma LPS, and the high-fat diet-induced metabolic disorders. Improved glucose tolerance, reduced body weight and fat mass development and inflammation were exhibited in both strains of obese mice. Another report by Brun et al. (2007) also indicated that plasma LPS is increased in leptin-deficient (*ob/ob)* and hyperleptinemic (*db/db)* mice. Similar findings were replicated in human studies which supported the findings of animal studies. Treatment of humans with antibiotic polymyxin B, which specifically targets gram-negative bacteria, successfully decreased LPS levels and eliminated hepatic steatosis (Pappo et al. 1991). Another report has shown that patients with type-2 diabetes had higher LPS levels than a well-matched group of control subjects without diabetes (Creely et al. 2006). Further investigation on role of gut microbiota in initiating the inflammatory reaction was carried out in healthy human subjects recently. Consuming a high-fat and low-carbohydrate diet for one month was significantly associated with increased plasma LPS level (71%), whereas low-fat diet reduced LPS level by 38% among healthy subjects (Pendyala et al. 2012).

ADVANCES IN ANALYSIS TECHNIQUES TO STUDYING THE DIVERSITY OF GUT MICROBIOTA

The decreasing cost, increasing speed and depth of DNA sequencing coupled with advances in the bioinformatics strategies provided several options to analyse microbiome using culture-independent method. Metagenomics, also known as 'environmental genomics' provides a powerful alternative to rRNA sequencing for analysing complex microbial communities (von Mering et al. 2007).

Metagenomic is an emerging field in which the power of genomic analysis applies to entire microbial communities through sequence-based and compositional analysis, without the need of isolating and culturing individual microbial species (Ventura et al. 2009). Qin et al. (2010) recently generated an extensive catalogue of DNA sequences from gut microbiota using metagenomics approach. They have characterized 3.3 million non-redundant microbial genes which were derived from 576.7 gigabases of sequences from European fecal samples.

More fecal metagenomic data were reported from Danish, Spanish and American to generate community-level metabolic networks of the microbiome. By categorizing metagenomics sequences based on gene functions, they constructed community-level metabolic networks varying in gene abundance and examined the topological features of these networks in relation to the phenotypes of the hosts. Their analysis identified specific network topologies related to obesity and inflammatory bowel disease (IBD) where lean and obese microbiomes differ primarily in their interface with the hosts and in the way they interact with the host metabolism (Greenblum et al. 2012).

Over the last decade, many investigations have focused on culture-independent approaches to evaluate the complexity of the intestinal microbiota. However, prior to the development of second generation sequencing technologies, the diversity and complexities of the uncultured organisms were constructed using the phylogenetic of 16S ribosomal RNA (rRNA) -based sequences (Gill et al. 2006; Pruesse et al. 2007; Ley et al. 2008).

These techniques however are dependent on PCR-based analysis of 16S rRNA as genome database of the majority of gut microbes have yet to be made available. Little information is available about microbial functions and the roles of microbiota in disease pathologies (Vacharaksa & Finlay 2010). Because of the limitations of these platforms, a wide variety of platforms have been developed to study gut microbial communities. Each of the platforms offers benefits and limitations (Table 1).

16S rRNA–based sequences have been used to analyze the diversity and complexities of the uncultured organisms. However, this technique does not provide direct evidence of functional capabilities (Gill et al. 2006; Pruesse et al. 2007; Ley et al. 2008).

Introduction of next generation sequencing (NGS) allows more extensive analysis of small-subunit 16S ribosomal RNA gene sequences and also WHOle genome shotgun sequencing of microbes to catalogue the bacterial genome (Turnbaugh et al. 2007; Shendure & Ji 2008; Gevers et al. 2012; Weinstock 2012). Since their introduction in 2005, NGS technologies can be classified into two main categories; PCR-based technologies and single-molecule sequencing (SMS) technologies (Shokralla et al. 2012) (Table 2).

To date, there are four commercially available NGS platforms adopting PCR-based technology; Roche 454 Genome Sequencer (Roche Diagnostics Corporation, Branford, CT, USA), AB SOLiD™ System (Life Technologies Corporation, Carlsbad, CA, USA), HiSeq 2000 (Illumina Inc., San Diego, CA, USA) and Ion Personal Genome Machine (Ion Torrent™) (Life Technologies, South San Francisco, CA, USA) (Shokralla et al. 2012).

For SMS technologies, three platforms have been developed and announced recently; HeliScope (Helicos BioSciences Corp., Cambridge, MA, USA), PacBio RS SMRT system (Pacific Biosciences, Menlo Park, CA, USA) and also Oxford Nanopore Technologies® (Oxford Nanopore Technologies Ltd, Oxford, UK) (Shokralla et al. 2012). The later platforms are considered the third generation sequencing technologies, which is non-PCR based and do

TABLE 1. Platforms used to study the gut microbiota

Platform	Method	16S rRNA based	Cost	Taxonomic resolution/ sensitivity	Advantages	Disadvantages (limitation)
Culture based	Multiplication of microbes in predetermined culture media under controlled laboratory conditions.	No	Very low cost	Moderate	Specific organism can be isolated. Functional information gained from what is known about the organism's substrate utilization and other physiological parameters.	Most of the gastrointestinal organism cannot be cultivated in current defined media.
Full-length Sanger Sequencing (Utilize the first generation of NGS technologies)	Fluorescent, dideoxy terminator	Full length sequenced with 2–3 reads	Expensive	High accuracy with long reads up to 750 bp	Sequencing the entire 16S gene maximizes the taxonomic resolution offered by the gene	Expensive and required an extensive bioinformatics analysis.
Metagenomics (Utilize second and third generation of NGS technologies)	Fluorescent-labeled nucleotides added simultaneously	Genome wide	Very expensive	High accuracy due to high coverage even though shorter reads (100 to 200 bp) in comparison to full-length Sanger sequencing	Provides a community-wide assessment of the functional genes present. 16S gene sequences provide taxonomic identification of community members. Sensitivity depends on the number of sequence reads obtained.	Shot-gun reads are mapped to reference genomes; this is limited by the number of sequenced genomes available. Extensive bioinformatics analysis required. Cloning biases could affect the functional gene information obtained. No direct information about which genes are expressed or functioning

Continued

TABLE 1. *Continued*

Platform	Method	16S rRNA based	Cost	Taxonomic resolution/ sensitivity	Advantages	Disadvantages (limitation)
Metabolomics	Simultaneous measurement of the levels of a large number of cellular metabolites.	No	Low in cost	Not able to identify the composition of gut microbiota directly. Pattern of metabotype associated with certain composition of microbiota can be obtained from metagenomics annotation server analysis derived from network metabolic construction of KEGG and SEED databases.	Metabolic profiles can be used to compare communities in a functional context. More direct functional information can be obtained. More rapid and less expensive than metagenomics. Non-targeted approach can also identify host metabolites associated with the gut microbiota.	No taxonomic information available. The direct correlation of type of metabolites produced by the microorganism is difficult to establish. Not all metabolites are detectable with current technology.

(TABLE 1 has been modified from Sekirov et al. 2010; Weinstock 2012; Ku & Roukos 2013)

not require amplification step prior to sequencing. SMS technologies were developed to provide a benchtop high-throughput sequencing platform suitable for a clinical setting. Modest set-up, effective running cost and rapid reads output were among quality that will be offered by SMS technologies (Loman et al. 2012; Shokralla et al. 2012).

Different NGS technologies have been used to dissect the diversity of gut microbiome and have their own advantages and limitations. The 454 pyrosequencing was the first NGS technology commercially available in 2005. This technology offers long reads length up to 800 base pair as compared with the rest of the NGS technology (Table 2) (Thomas et al. 2012; Weinstock 2012). Long reads generated from this 454 pyrosequencing offered more flexible output for accurate data binning and annotation in metagenomic analysis (Weinstock 2012). However, reads generated from this technology are prone to have insertion-deletion errors in homopolymers regions with high replication of sequences.

Error as such is caused by the faulty CCD camera in 454 which translate the actual number of incorporated nucleotides with their exact position during polymerization. Incorrect translation is due to the low intensity or too many variations of emitted light produced during sequencing by synthesis. Sequences with this frameshift might be interpreted as a rare biota in bioinformatics analysis, especially for protein prediction and annotation using KEGG or SEED pathway. Incorrect prediction or annotation happens whenever protein coding sequences (CDSs) are called on a single read that contained the frameshifts (Thomas et al. 2012).

The major advantage of Illumina and SOLiD technologies as compared to 454 technologies is that the earlier detect each of the nucleotide incorporated one at a time during polymerization step (Shokralla et al. 2012; Thomas et al. 2012; Weinstock 2012). In illumina, each cluster of templates is supplied with polymerase and four differently labelled fluorescent nucleotides that have their 3'-OH chemically inactivated. After each nucleotide is incorporated, excitation of fluorescent is detected by the system to identify the incorporated nucleotide. The additional step of chemical deblocking treatments will remove the fluorescent group and this allows the flowing nucleotides to be incorporated with the new fluoresced nucleotides labelled in the next flow cycle (Shokralla et al. 2012). This specific one oligo per flow cycle with deblocking treatment minimize framshifts problems in generating sequences as compared with 454 pyrosequencing in both 16S rRNA and shotgun metagenomics sequencing.

Even though SOLiD technology applies emulsion PCR similar with 454 pyrosequencing, it uses a sequencing-by-oligo ligation technology to ensure homopolymer region is accurately sequenced (Shokralla et al. 2012). This technology will attach the universal adaptors-linked DNA fragments with complementary oligo bases present on the surface of each 1-mm magnetic bead.

By this, starting of every DNA fragment is both known and identical before these magnetic beads are amplified individually by emulsion PCR. The resulting amplified sequences attached to the magnetic beads will be covalently bonded to a glass slide. This platform also employs the same deblocking treatment used by Illumina technologies prior to the following incorporation or ligation of nucleotide during polymerization.

However, one of the disadvantages of both Illumina and SOLiD systems is their relatively short reads length ranging between 35 bp to 200 bp as depicted in Table 2. For 16S rRNA sequencing strategy, 454 pyrosequencing is more favoured by scientists since the longer radius of up to 800 bp offered by this platform is able to cover up to three variable regions of 16S per reads. Shorter reads generated from Illumina and SOLiD sequencer is able to cover only one variable region of 16S (Weinstock 2012). For phylogeny analysis, several regions of 16S are needed since short reads limits its application for alignment, assignment and annotation in downstream analysis.

For shot-gun approach, PCR-based NGS technologies are sharing the same problem in which bias are introduced during amplification. It may happen in two stages as reviewed by Shokralla et al. (2012), the first incidence may occur during library preparation due to low template concentration, incorrect primer selection and un-optimal profile of annealing temperature and number of replication cycles. In addition, bias can be introduced during library amplification by emulsion or bridge PCR prior to sequencing (Shokralla et al. 2012).

Ion Torrent and more recently Ion Proton which was launched in 2010 and 2012 respectively, are the other 2 platforms offered as a second generation of NGS technologies (Shokralla et al. 2012). Both technologies are based on detection of hydrogen ion releases as a by-product during nucleotide incorporation of DNA polymerization. This technology uses ion semiconductor chip, an array of micro wells chips contains an ion sensor beneath which detects changes in the concentrations of hydrogen ion whenever nucleotides is incorporated. As up to date, there are three different micro wells offered by Ion Torrent; 314, 316 and 318 million chips, which are able to generate reads up to 1 Gb (Table 2).

The more advanced sequencer is the Ion Proton II, which contains 660 million micro wells to capture the release of hydrogen ions during DNA polymerization (Shokralla et al. 2012; Thomas et al. 2012; Weinstock 2012). The generated reads data is approximately 100-fold more massive than Ion Torrent. This platform provides an alternative for scientists to do either 16S rRNA or shotgun metagenomics sequencing since it offers reads up to 400 base reads per run as compared to Illumina and SOLiD technologies.

To overcome this bias and short read problem from PCR based technologies sequencers, the third generation technology employs SMS technologies to bypass the needs of amplification prior sequencing. Helico Heliscope,

TABLE 2. Current available platform use in high-throughput sequencing technologies

Phase	Category	Platform	Method	Read length (bp)	Max. no of reads/run	Sequencing output/run	Run Time
Second generation	PCR-based NGS technologies	Roche 454 GS FLX	Each nucleotide incorporated during DNA polymerization will release a pyrophosphate molecule which will produce a light through reactions with enzyme luciferase. The emitted light will be captured by CCD camera in the sequencer	400-500	1×10^6	≤ 500 Mb	10 h
		Roche 454 GS FLX+		600-800	1×10^6	≤ 700 Mb	23 h
		Roche 454 GS Junior		400-450	1×10^5	~35 Mb	10 h
		Illumina HiSeq 200	Fluorescent, sequencing by synthesis, bridge amplification PCR.	100-200	6×10^9	≤ 540-600 Gb	11 d
		Illumina HiSeq 1000		100-200	3×10^9	≤ 270-300 Gb	8.5 d
		Illumina GAIIx		100-200	6.4×10^8	≤ 95 Gb	7.5-14.5 d
		Illumina MiSeq		100-150	7×10^6	≤ 1-2 Gb	19-27 h
		AB SOLiD 5500 system	Sequencing-by-oligo ligation technology, emulsion PCR	35-75	2.4×10^9	~100 Gb	4 d
		AB SOLiD 5500 xl system		35-75	6×10^9	~250 Gb	7-8 d
		Ion Torrent – 314 chip	Ion semiconductor sequencing by synthesis, real time detection of hydrogen ions / proton released during the polymerization of DNA	100-200	1×10^6	≥ 10 Mb	3.5 h
		Ion Torrent – 318 chip		100-200	6×10^6	≥ 100 Mb	4.7 h
		Ion Torrent – 318 chip		100-200	11×10^6	≥ 1 Gb	5.5 h
		Ion Proton I		100-200	8×10^7	Up to 10 Gb	2-4 h
		Ion Proton II		Still under development			
Third generation	SMS technologies	Helicos Heliscope	Detects incorporation of fluoresced nucleotide on individual strands without amplification	30-35	1×10^9	~20-28 Gb	≤1 d
		Pacific Biosciences system	Detects fluoresced moiety of phosphor-linked nucleotides during polymerization of DNA	≥1500	50×10^3	~60-75 Gb	0.5 h
		Oxford Nano Technologies	Use of electronic chip to measure changes of electrical current in the nano membrane as DNA passes through pore, signal will be decoded to identify the sequences of passed bases	200-300	Long reads	Still under development	Still under development

(TABLE 2 has been modified from Shokralla et al. 2012; Weinstock 2012)

PacBio and Oxford Nanopore are the examples (Table 2). Helicos Heliscope was the first SMS sequencer available in the market back then in 2008. No amplification is needed after the library construction, where DNA polymerase and four fluorescently labelled nucleotides will be flowing in repetitively as the strands of DNA is extended. This platform offers read nearly 1 billion sequenced reads per run (Table 2).

PacBio or Oxford Nanopore focuses on the use of 16S rRNA and shotgun sequences, aiming to amplify more than 200 base reads on one go (Table 2). PacBio is offering long sequencing reads of up to 10 kb. This technology uses real time single molecule sequencing approach in which fluoresced light pulses, emitted as a byproduct of nucleotide incorporation during sequencing are recorded.

However, low accuracy of reads generated by PacBio sequencer is one of the drawbacks in metagenome analysis (Weinstock 2012). Oxford Nanopore is another latest single molecule based sequencing platform, developed by Oxford Nanopore Technologies Consortium. This platform targets to sequence very long reads, using detection on electronic signal produced whenever the nucleotide passes through a nanopore membrane. However the accuracy of this platform on 16S rRNA and shotgun sequencing for microbiome analysis is not guaranteed since it is still under development and trial (Weinstock 2012).

Besides genomic-based technology, the non-genomic approach such as metabolomics is a useful platform to study the metabolic activity of complex microbial populations through analysis of their metabolic profiles. The gut microbiota is believed to communicate with the host via a characteristic pattern and thus participate in the host metabolic network. The advances of profiling techniques such as 1H nuclear magnetic resonance (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) allow the simultaneous monitoring of changes in metabolites with diverse chemical properties and at a wide range of concentrations (Griffin 2006; Mashego et al. 2007). Metabolomics may provide clearer picture on the relationship between microbiota and its metabolisms, with host's metabolisms and diseases. However, due to high complexity of most body fluids and tissues, a comprehensive view of all the metabolites present in a sample is still not possible (Sekirov et al. 2010).

Application of mass spectrometry based-metabolomics in characterizing the impact of the murine intestinal microbiota showed that gut microbiota is essential for the production of bioactive indole-containing metabolites such as the antioxidant indole-3-propionic acid from tryptophan. Thus, this finding suggested that the gut microbiota has a profound and systemic impact on host metabolism (Wikoff et al. 2009). A more recent study has analyzed the colonic luminal metabolome using a novel technique, capillary electrophoresis mass spectrometry with time-of-flight (CE-TOFMS). A total of 179 metabolites were detected from the colonic luminal metabolome. Meanwhile, 48 metabolites were detected in significantly higher concentrations in germ free mice compared to ex-germ free mice. The colonic luminal metabolome is highly influenced by gut microbiota and a comprehensive catalogue of intestinal luminal metabolome (host and bacteria) is essential in order to understand the effects of host-intestinal bacterial interactions (Matsumoto et al. 2012). Schematic diagram on how metagenomic analysis and metabolomics analysis could discerning the role of gut microbiota in diseases is depicted in the Figure 1.

COMPUTATIONAL ANALYSIS DISSECTING THE GUT MICORBIAL ECOLOGY

Data from the metagenome analysis are vast and rich with information generated from various platforms as discussed earlier. According to Weinstock (2012), metagenomic data analysis has three phases depending on either it is 16S rRNA gene or shotgun sequencing. At the first phase of both sequencing, reads produced must be in a good quality, to avoid any misclassification during taxonomic analysis. Issue on chimeras sequences, read length after removal of low quality bases, duplicates reads, and also contamination of human sequences must be addressed before further analysis is performed.

In the second phase, sequences generated from 16S rRNA gene sequencing can be classified based on taxonomic or clustering using Operational Taxonomic Unit (OTU) (Weinstock 2012). For taxanomic classification, the sequences generated are compared with the existing bacterial 16S rRNA gene databases such as Ribosomal Database Project (RDP), Greengenes, SILVA, and GAST.

Taxonomy-supervised analysis will be able to classify bacterial strains according to similarity in physiology, morphology and any genetic constituents. OTU clustering system classifies or clusters the sequences of a closely related species with a 97% homology using alignment-based clustering. This specific nucleotide homology represents a community relationship between bacteria which is based on nucleotide distance using 16S rRNA gene sequences (Woo-Jun et al. 2011).

Databases for bacterial 16S rRNA are available in GenBank or Kyoto Encyclopedia of Genes and Genomes (KEGG) and are useful in comparative analysis. Data obtained from shot-guns reads can be compared with their respective species using a simple Basic Local Alignment Search Tools (BLAST). This alignment uses a specified homology percentage of 97% to generate a list of genes that were matched reads from these two databases (Weinstock 2012). However, there are limitations currently faced by researchers as not all bacteria has a deposited reference genome and in some cases, and some new roads are matched to the genes whereby the function has not been elucidated.

A variety of software can be used in the second phase of analysis, for base-calling and detection of polymorphism of the sequences generated, de novo assembly using paired

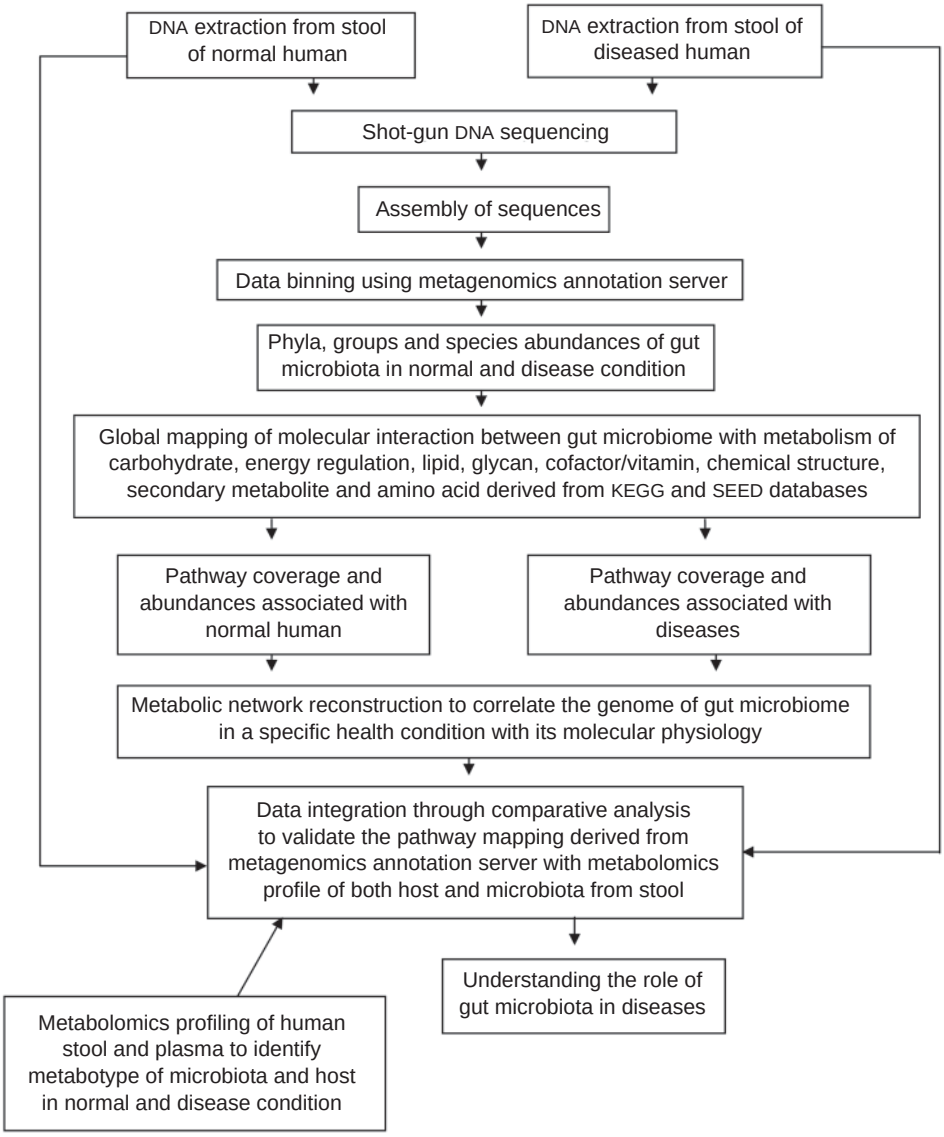

FIGURE 1. A flow chart showing the network of metagenomics and metabolomics in discerning the role of gut microbiota in diseases

or unpaired reads, annotation and prediction analysis. To be specific, BLAST is a suitable tool to align long reads, whereas Short Oligonucleotide Analysis Package (SOAP) de Novo can be used to align and reconstruct short reads without the aid of a reference genome. The assembly of these short fragments of sequences is very challenging since these reads have relatively low accuracy, thus de novo mate-paired reads assembly is the best alternative (Shendure & Ji 2008; Weinstock 2012).

In the third phase of analysis, the assembled reads of similar communities were plotted on the abundance curves or biodiversity plots to generate the rarefraction curve of the studied metagenome (Schloss et al. 2009; Lozupone et al. 2011). Then, functional aspect of metagenome will be described to understand the roles of microbiota. One of the software that could be used is HMP Unified Metabolic Analysis Network (HUMAnN) (Abubucker et al. 2012). Using this software, the shot-gun reads will be aligned to KEGG ontology database using the BLAST search hit list. The assignment of each gene family for each shot-gun reads are based on the total sums of the alignment from the BLAST search hit list. The identified gene will be assigned to metabolic pathway using MinPath feature available in this software. The identified pathway will be reconstructed using a maximum parsimony method with several filtrations to remove false positive pathway and to account for rare genes in abundant pathways. The resulting output will provide information on the presence or absence and abundances of the identified pathways (Abubucker et al. 2012). Therefore, the role of microbiota on the physiology and homeostasis of the host could be understood.

Through genes weighted sum of hits, a list of hits from the BLAST search linked with KEGG and SEED databases is used to create metabolic pathways in order to reconstruct the functional descriptions of community (Abubucker et al. 2012). Variance analysis can be done through alignments

of DNA reads to reference genomic. Computational analysis can also be used to determine which organisms co-occur or rarely co-occur by assessment of the dynamics of community structure in longitudinal time series (Caporaso et al. 2011).

For analyzing metabolomics data, many computational tools have been developed. Various metabolites produced by gut microbiota can be analyzed using Mass Profiler Professional (MPP) software from Agilent Technologies (Agilent information 2012) and the databases such as METLIN Metabolite Personal Compound Database (PCD) and the METLIN Metabolite Personal Compound Database and Library (PCDL) provide information on the biological activities of small molecules (Agilent information 2012). The characterization of gut microbiota metabolic fingerprint and its interaction with the host can be revealed through metabolomics approach (Marcobal et al. 2013). The established biological pathways analysis are performed using Pathway Architect to efficiently project the results of differential abundance results onto publicly available biological pathways, including KEGG, BioCyc and WikiPathways (Scalbert et al. 2009; McHardy et al. 2013).

CONCLUSION

The gut microbiota is increasingly being accepted as an environmental factor that affects host metabolism and contributes too many chronic pathological conditions such as obesity, diabetes and cardiovascular disease. Compelling evidence supports the concept that the microbial community participates in the development of the fat mass deposition, insulin resistance and low-grade inflammation that characterizes obesity. The development of powerful analytical methods will provide novel data lending insight into the complexity of the gut microbiota. Nevertheless, more researches with advanced methods should be carried out in order to determine how specific changes in the gut composition will affect or counteract the development of diseases.

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