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REVIEW

Upper gastrointestinal microbiota and digestive diseases

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Abstract

Metagenomics which combines the power of genomics, bioinformatics, and systems biology, provide new access to the microbial world. Metagenomics permit the genetic analysis of complex microbial populations without requiring prior cultivation. Through the conceptual innovations in metagenomics and the improvements in DNA high-throughput sequencing and bioinformatics analysis technology, gastrointestinal microbiology has entered the metagenomics era and become a hot topic worldwide. Human microbiome research is underway, however, most studies in this area have focused on the composition and function of the intestinal microbiota and the relationship between intestinal microbiota and metabolic diseases (obesity, diabetes, metabolic syndrome, *etc.*) and intestinal disorders [inflammatory bowel disease, colorectal cancer, irritable bowel syndrome (IBS), *etc.*]. Few investigations on microbiota have been conducted within the upper gastrointestinal tract (esophagus, stomach and duodenum). The upper gastrointestinal microbiota is essential for several gastrointestinal illnesses, including esophagitis, Barrett's esophagus, and esophageal carcinoma, gastritis and gastric cancer, small intestinal bacterial overgrowth, IBS and celiac disease. However, the constitution and diversity of the microbiota in different sections of the upper gastrointestinal tract under health and various disease states, as well as the function of microbiota in the pathogenesis of various digestive diseases are still undefined. The current article provides an overview of the recent findings regarding the relationship between upper gastrointestinal microbiota and gastrointestinal diseases; and discusses the study limitations and future directions of upper gastrointestinal microbiota research.

Key words: Microbiota; Upper gastrointestinal tract; Digestive diseases; 16S rDNA; Metagenomics

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INTRODUCTION

Microbes associated with the human body include bacteria, archaea, fungi, and viruses. The vast majority of studies on microbiota have focused on bacteria. A large number of micro-organisms colonize on the surface of and within the human body and can reach counts of 10^{12}-10^{14}, 10 times that of the cells of the human body, while the number of genes in these micro-organisms, approximately 3.30 million, is 150 times higher than that in the human body[1]. Humans are a type of super organism composed of the human body and symbiotic micro-organisms[2], therefore, researching human diseases from only the human body point of view reveals only a partial view of a condition; thus, the role of commensal microbiota in human health and disease must be considered. Micro-organisms and their metabolites play important roles in human energy metabolism[3], the absorption of nutrients[4], immune function[5] and other important physiological activities[6]. In fact, a variety of human diseases will be induced when commensalism between the

host and the microorganisms is disrupted.

Microbiota composition has classically been analyzed using cost-effective and reproducible culture techniques that use differential media to select for specific bacterial populations. Early microbiological studies relied mainly on traditional culture-based methods. However, the overall microbial community structure, spatial distribution and dynamics could not be fully demonstrated using traditional culture-based methods due to the fact that 99% of such micro-organisms are uncultured[1]. As the gene sequence of 16S rRNA has a combination of highly conserved and variable sequences in a relatively short portion of the bacterial chromosome[7], it is increasingly used to characterize the diversity of the complex microbial communities that can be sampled from different sites of the body in both healthy individuals and patients with diverse pathological conditions[8-12]. Many bacterial *16S rRNA* gene-dependent methods, including terminal restriction fragment length polymorphism (TRFLP)[13], denaturing and temperature gradient gel electrophoresis (DGGE and TGGE)[14], ribosomal intergenic spacer analysis (RISA)[15], DNA microarray[16] and fluorescence *in situ* hybridization (FISH)[17], have emerged as molecular biology techniques; however, the information obtained by the above methods has been very limited, thus, ongoing studies cover only a small portion of this field.

Recent microbiological studies are firmly supported technically along with the introduction of the concept of metagenomics[18], the development of high-throughput DNA sequencing and bioinformatics technology. Metagenomics mainly include two strategies: first, the high-throughput sequencing of 16S rDNA hypervariable regions, which can provide diversity and abundance information on microbial communities, while not supplying the functional genes of microbiota and that is mainly used in the classification, identification and comparison of microbiota[8,9]; second, metagenomic sequencing of whole community DNA, not only provides information on microbiota structure and abundance, but can also be used in the functional annotation and building of metabolic networks, and favors the in-depth investigation and screening of functional genes[1,19,20]. Over the past 3-5 years, sequencing cost and time have been greatly reduced; thus, the feasibility of comprehensive and detailed studies of the microbiota has increased significantly.

The United States National Institutes of Health launched the Human Microbiome Project (HMP) in 2007[20]. The European Commission also initiated the metagenomics of the human intestinal tract funded by the 7th Framework Program of the European Union in 2008. Scholars from various countries established the International Human Microbiome Consortium in 2009, an international cooperative effort aiming to explore the relationship between microbiota and human health and disease. The editors of *Science* had predicted that human microbe research may become a new hot topic worldwide[2]; in fact, studies of microbiota in human health and disease based on high-throughput sequencing and bioinformatics technology have already been initiated. Not long ago, the HMP Consortium investigated the human microbiome based on healthy adults sampled from five body regions including the feces, oral cavity, airway, skin and vagina, which generated 5177 microbial taxonomic profiles from *16S ribosomal RNA* genes and > 3.5 terabases of metagenomic sequence to date[19]. However, the mucosa-associated microbiota of the gastrointestinal tract was not evaluated in this study. Most researches today have focused on intestinal microbiota and the relationship between intestinal microbiota and metabolic diseases (obesity, diabetes, metabolic syndrome, *etc.*)[1,21-23] and intestinal disorders [inflammatory bowel disease (IBD), colorectal cancer (CRC), irritable bowel syndrome (IBS), *etc.*][1,24-26], and rarely examined upper gastrointestinal tract microbiota. Here we review the evidence for a microbiota concept in upper gastrointestinal diseases and highlight recent studies that have enhanced our appreciation of the relationship between microbiota and human health and disease.

MICROBIOTA AND THE ESOPHAGUS

The esophagus, unlike the oral cavity, stomach and colon, does not retain food contents. Studies using culturing methods have suggested that the esophagus is either sterile or contains only a few transient microbes originating from the oropharynx by swallowing or from the stomach by gastroesophageal reflux[27]. Moreover, under certain disease conditions, several pathogenic microorganisms, such as *Candida albicans*, *Cryptococcus* or *Herpesvirus,* can infect the esophagus[28-31]. Whether an imbalance of esophageal microbiota is responsible for esophageal disorders remains unclear; in fact, investigations of esophageal microbiota remain limited[27,32-38].

Culture-based studies mainly used luminal washes of esophageal contents[27,36] and their results were not convincing. Gagliardi *et al*[27] demonstrated that *Streptococcus viridans* (*S. viridians*) may be the most numerous microorganism in both the normal esophagus and the oropharynx. Norder Grusell *et al*[36] also reported that *S. viridians* was the most common bacterium using both brush samples and biopsies; and this study confirmed that the human esophagus could be colonized with a resident flora of its own, although it was similar to the flora present in the oral cavity.

Culture-independent methods have recently been used more frequently to characterize the diversity of the microbiota in the esophagus[33,37,38]. Pei *et al*[33] investigated the composition of microbiota in the normal distal esophagus using broad-range 16S rDNA polymerase chain reaction (PCR). They confirmed that the majority of esophageal microbiota were known and cultivable; and found that *Streptococcus*, *Prevotella* and *Veillonellance* were the most prevalent genera in esophageal biopsies. Yang *et al*[37] characterized the diversity of the microbiota of the distal esophagus in individuals with normal esophagus and in patients with esophagitis and Barrett's esophagus using

16S rDNA sequencing. They classified the esophageal microbiota into two types: *Streptococcus*-dominated in the normal esophagus and *Gram-negative anaerobes* in Barrett's esophagus and esophagitis[37]. However, this study could not answer the question of how the microbiota participates in the pathogenesis of esophageal inflammation.

Gastroesophageal reflux impairs the mucosal barrier in the distal esophagus, allowing chronic exposure of the epithelial cells to diverse microbiota and inducing chronic inflammation. Chronic inflammation may play a critical role in the progression from reflux-related intestinal metaplasia or Barrett's esophagus to esophageal carcinoma[39]. Until now, there has been no research on the diversity of esophageal microbiota in patients suffering from esophageal squamous or adenocarcinoma.

Interestingly, Fillon *et al*[40] sampled the microbiome in normal histological esophageal mucosa using a novel device, the Enterotest™ capsule, and found that the microbiota phylum-level diversity was similar in samples from the esophageal mucosa biopsy, esophageal string test, oral string and nasal swab as identified using 454 pyrosequencing; moreover, at the genera level, the most common three genera, *Streptococcus*, *Prevotella*, and *Veillonella,* were similar in the esophageal string test and mucosal biopsy samples, a finding that was consistent with the findings of Pei *et al*[33]. In fact, this novel instrument could be used for future research on the microbiota within the human esophagus. However, this study did not eliminate the effect of proton pump inhibitors (PPI)[41,42], steroids[43] or restricted diet on esophageal microbiota. It is also evident that PPI could have an effect on gastrointestinal microbiota[41], and long-term PPI treatment is very common in reflux esophagitis, therefore, it may be meaningful to estimate the effect of long-term PPI treatment on esophageal microbiota.

In addition, Chagas' megaesophageal disease caused by *Trypanosoma americanum* infection is usually associated with esophageal bacterial overgrowth, recurrent pulmonary infections and esophageal neoplasia[32]. Pajecki *et al*[32] showed that patients with megaesophageal disease could present with a wide variety of microbiomes, mainly aerobic *Gram-positive* and *anaerobic bacteria*. The imbalance of esophageal microbiota could play a causal role, and high-throughput sequencing technology could be used to understand esophageal bacterial overgrowth in Chagas' megaesophageal disease.

MICROBIOTA AND THE STOMACH

The stomach is a special area in the gastrointestinal microecosystem. Its unique ecological environment and characteristic microbial community are due to gastric acid secretion. Because the stomach connects the esophagus and oral cavity on the upper side and the duodenum on the lower side, bacteria from the mouth, pharynx, nose, respiratory tract, esophagus and small intestine can enter the stomach. It was once believed that gastric acid could kill the bacteria entering the stomach and that the stomach environment was not suitable for bacterial colonization; however, some studies using traditional culture methods confirmed that large numbers of acid-resistant bacterial strains exist in the stomach and are mainly derived from the transient flora in the mouth and food, including *Streptococcus*, *Neisseria* and *Lactobacillus*, while the content was generally < 10^3 colony-forming unit/mL (CFU/mL)[44].

In 1984, Marshall *et al*[45] isolated *Helicobacter pylori (H. pylori)* from the stomach, thus starting a new era of *H. pylori* and digestive diseases research, and won the Nobel Prize in medicine. With the development of molecular biology and bacterial 16S rDNA identification techniques, the composition of the stomach flora was gradually investigated using new molecular biological methods. Using bacterial 16S rDNA PCR and TGGE, Monstein *et al*[46] demonstrated that some other microbes, including *Enterococcus*, *Pseudomonas*, *Staphylococcus* and *Stomatococcus,* exist within the gastric mucosa.

The identification of stomach flora has increased dramatically with the development of metagenomics and high-throughput sequencing technology. Bik *et al*[47] performed a 16S rDNA sequencing analysis of the stomach flora of 23 patients with gastric diseases, identified 128 kinds of phylotypes belonging to eight classes, and obtained 1056 non-*H. pylori* clones. Li *et al*[48] performed a 16S rDNA high-throughput sequencing analysis of the gastric mucosa-associated flora in *H. pylori*-negative patients with gastritis who had never used non-steroidal anti-inflammatory drugs and obtained a total of 1223 non-*H. pylori* clones that could be classified into 133 kinds of phylotypes belonging to eight bacterial classes. Although the above two studies were conducted in populations within different regions and ethnic groups, the stomach flora composition was very similar among those populations. The two studies identified approximately 130 phylotypes belonging to 7-8 classes, and 77.4% and 79.8% of clone fragments separately for each study were homogeneous. The two species with the highest abundance, *Streptococcus* and *Prevotella*, were the same. Andersson *et al*[49] conducted pyrosequencing analyses of gastric mucosa-associated flora in six healthy subjects and obtained 262 phylotypes belonging to 13 classes, including strains that had not been confirmed by other studies such as *Chlamydia* and *Cyanobacteria*. Stearns *et al*[50] performed high-throughput 16S rDNA sequencing analysis of the oral, stomach, duodena and colon mucosa-associated flora as well as feces-related flora in four healthy subjects. The obtained stomach flora constitution was more detailed than that obtained by Bik *et al*[47] due to the higher sequencing depth. The high-throughput 16S rDNA sequencing techniques based on the metagenomics strategy were adopted in the above studies; thus, the obtained stomach flora information was detailed and showed that huge numbers of bacteria other than *H. pylori* exist in the stomach.

Gastrointestinal microbiota distribution is spatially specific. The gastric juice-associated microbiota is easily

affected by diet and other factors; thus, they are variable. In contrast, the gastric mucosa-associated microbiota is relatively stable and less affected by interference factors. Furthermore, gastric mucosa-associated flora can affect the host more directly and are more closely related to the pathogenesis of gastric disease. Li *et al*[48] thoroughly washed gastric mucosal biopsy samples, but found no change in the constitution of gastric mucosa-associated flora; thus, the gastric flora proved to be closely associated with gastric mucosa and could not be readily washed away. Although some microbes such as *Streptococcus* genus have a high abundance in both the oral cavity and stomach[47,48,51], the study by Li *et al*[48] indicated that many non-*H. pylori* microbes could be resident flora in the human stomach, and not just transient flora from the oral cavity.

The use of feces has been widely adopted in the study of the relationship between intestinal microbiota and diseases as it is easily sampled and reflects the overall constitution of the intestinal flora. It is worth noting that feces-associated flora are less interfered with by host DNA; thus, they can be sequenced for whole communities of DNA or for 16S rDNA hypervariable regions. Gastrointestinal mucosa-associated flora are usually interfered with by host DNA; thus, they are commonly investigated using 16S rDNA sequencing.

Stomach flora and H. pylori

H. pylori is a *Gram-negative bacillus* that colonizes the stomachs of approximately 50% of individuals worldwide. *H. pylori* has been investigated more deeply than any other stomach pathogen. Although *H. pylori* is the major pathogenic factor of chronic gastritis and peptic ulcer and is one of the risk factors for gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma[52,53], the relationship between *H. pylori* infection and gastric diseases remains unclear. Stomach colonization was negative even after several weeks of oral implantation of *H. pylori* in specific pathogen-free animal models; however, it was usually positive in germ-free animal models[54], suggesting that other stomach flora affected the intragastric colonization of *H. pylori* and its activity in gastric diseases.

In vivo and *in vitro* studies based on animal models have found that some probiotics including *Lactobacillus*, *Bifidobacterium* and *Saccharomyces* can prevent the adhesion, colonization and growth of *H. pylori* in gastric mucosa. Zaman *et al*[55] confirmed that some *Lactobacillus* species prevented the colonization of *H. pylori* in the stomachs of Mongolian gerbils, while *Eubacterium limosum* promoted the colonization of *H. pylori*. Yin *et al*[56] found that long-term intragastric colonization of *H. pylori* could affect the distribution and number of flora in the stomach and duodenum. For example, the propagation of *Lactobacilli* was inhibited, while *Enterococcus*, *Staphylococcus aureus*, *Bifidobacterium* and *Bacteroides* were rarely affected. Sun *et al*[57] believed that some *Lactobacillus* species were dominant strains in the stomachs of Mongolian gerbils that were not affected by *H. pylori* infection; *Lactobacillus gasseri* and *Lactobacillus reuteri* existed in the gerbils' stomachs and suppressed the colonization and growth of *H. pylori*.

A small number of studies based on the human body also confirmed that *H. pylori* interacted with other gastric flora. Using traditional culture methods, Garcia *et al*[58] analyzed the gastric mucosa-associated flora and showed that some *Lactobacillus* species competed with *H. pylori* for colonization resources. Hu *et al*[59] conducted bacterial cultures using gastric mucosal biopsy samples and found that the dominant species, including *Streptococcus*, *Neisseria*, *Rothia* and *Staphylococcus*, were primarily sourced from the upper respiratory tract. They believed that *H. pylori* infection was usually accompanied by the colonization of non-*H. pylori* bacteria; they noted that these non-*H. pylori* bacteria played certain roles in gastric diseases. Sanduleanu *et al*[60] thought that non-*H. pylori* bacteria and their products could persist in the stomach as antigenic stimulators that enhance the immune response caused by *H. pylori* infection and that their co-infection could promote the development of atrophic gastritis. Clinical trials have initially confirmed that probiotics treatment can reduce the gastric colonization density of *H. pylori*[61]. Probiotics can increase the eradication rate, decrease the relapse rate and reduce antibiotic side effects; thus, they may be used as an effective supplement in *H. pylori* eradication therapy.

The above results suggested that the *in vivo* colonization of *H. pylori* was closely related with other stomach flora; however, most of the above studies were based on animal models and limited by their methods. As such, composition of the human stomach flora and which stomach flora can inhibit or promote the intragastric colonization of *H. pylori* have not been or cannot be comprehensively understood.

Stomach flora and gastric cancer

Gastric cancer is one of the most common malignancies worldwide. The pathogenesis of gastric cancer is a process of multiple stages and steps affected by multiple factors that involve a huge number of molecules and complex regulation networks. The cause of gastric cancer is still not clear despite deep decades-long studies. It is generally believed that environmental, dietary, *H. pylori* infection and genetic factors participate in the pathogenesis of gastric cancer. Furthermore, *H. pylori* is closely related with gastric cancer, although whether other intragastric flora facilitate or inhibit the effect of *H. pylori* in gastric cancer development remains unknown and very few studies have examined this issue.

The pathogenesis of CRC may be the result of interactions among the intestinal flora, intestinal mucosal immunity and host genetic susceptibility[62]. Intestinal flora interfere with the signal mechanisms of pro-inflammatory reactions in the intestinal mucosa and results in excessive repair of mucosal injury and ultimately induces tumorigenesis and canceration[63]. Some intestinal microbes and their metabolites have direct or indirect cytotoxic effects on intestinal mucosal epithelial cells, and incomplete repair of damaged intestinal mucosal epithelium may result

in neoplastic transformation[63]. Animal studies have also confirmed that permanent and normal intestinal flora are necessary for intestinal tumorigenesis[64]. Studies on the relationship between intestinal flora and CRC indicated that the permanent and normal intragastric flora and their composition might participate in the pathogenesis of gastric cancer. However, few studies have focused on the issue of whether new ways of enabling early warning and early diagnoses of gastric cancer can be established using in-depth analysis and studies of gastric flora composition. The primary reason for this is that due to the large and complex flora network, comprehensive and detailed information on flora constitution could not be obtained by studies using culture-based methods; thus, the microecological study of the relationship between other stomach flora and gastric cancer pathogenesis could not be conducted. Dicksved *et al*[65] recently analyzed the stomach flora constitution of patients with gastric cancer and found no significant differences in the stomach flora of patients with gastric cancer and those of patients with dyspepsia and normal gastric mucosa. However, there were many limitations in that research, including the small size of the included samples and the fact that a new generation of high-throughput sequencing technology was not used; thus, the results remain to be confirmed since the stomach flora were not comprehensively and deeply studied. To date, no study on the relationship between stomach flora and gastric cancer using high-throughput sequencing technology based on the metagenomics strategy has been performed.

Stomach flora and gastric polyp

Gastric polyps are focal elevated lesions within the gastric epithelium mucosa. The current limited systematic studies of gastric polyps focus mainly on the relationship between gastric polyps and cancer as well as the role of *H. pylori* in the pathogenesis. The possible pathogeneses related to the development of gastric polyps include hereditary factors, bile reflux, *H. pylori* infection, etc., while none have been directly proved. As such, the etiology and biological characteristics of gastric polyps and its long-term effects on the human body are not yet clear. Studies of gastric polyps are far less detailed than those of colonic polyps. The intestinal flora are involved in the pathogenesis of colonic polyps[24,25], while no study focusing on the relationship between stomach flora constitution and gastric polyps pathogenesis from the perspective of gastric microbiota using bacterial 16S rDNA sequencing has been reported.

Overall, the constitution and diversity of stomach flora under various disease states, the interactions between *H. pylori* and other stomach flora and their underlying mechanisms as well as the effect of stomach flora in the pathogenesis of various stomach diseases are expected to be uncovered more deeply from the perspective of intestinal microbiota using high-throughput bacterial 16S rDNA sequencing technology based on the metagenomics strategy.

MICROBIOTA AND THE DUODENUM

Much less is known about the microbes that are present within the duodenum, particularly because collecting samples for such microbial ecology studies is much more challenging. However, continued efforts in this regard are needed, especially in light of the growing recognition of the composition of the duodenal microbiota and the association with health and gastrointestinal disorders as revealed in recent studies. Duodenal microbiota studies have focused predominantly on small intestinal bacterial overgrowth (SIBO), IBS, and celiac disease (CD).

Duodenal flora and SIBO

Some risk factors, such as demographics (older age), anatomic abnormalities (*e.g.*, small intestinal diverticula and gastric resection), motility disorders (*e.g.*, CD, diabetic neuropathy and scleroderma), organ system dysfunction (*e.g.*, cirrhosis, chronic pancreatitis and end-stage renal disease), and medications (*e.g.*, recurrent antibiotics and gastric acid inhibitors), are closely associated with SIBO[66].

SIBO has been traditionally defined according to the number and type of culturable bacteria within duodenal or jejunal aspirates: 10^5 CFU/mL of colonic-type bacteria has been commonly used[67]. Although some studies have diagnosed SIBO using a direct test-that is, bacterial cultures of aspirate from the small bowel[68], the majority of gastrointestinal microbiota could not be cultured, the culture-based method can not reveal the real changes in microbiota in the small intestine in various disease conditions. The lactulose/glucose breath tests have also recently been used for the diagnosis of SIBO[69,70], however, these are indirect tests with poor sensitivity and specificity[71,72]. In the future, high-throughput bacterial 16S rDNA sequencing may be used to determine the composition of microbiota in the small intestine in some disorders which could lead to SIBO, and contribute to new diagnostic criteria for SIBO.

Duodenal flora and IBS

IBS is a common disorder characterized by abdominal pain or discomfort associated with disturbed bowel function such as constipation and/or diarrhea[73]. Epidemiological and clinical data support the new bacterial concept of IBS[74]. Altered intestinal microbiota composition[75-79] and gut flora metabolites (*e.g.*, short-chain fatty acids butyrate, acetate, and propionate; CH_4 and H_2 gases)[79,80] were observed in patients with IBS. An etiological role of gastrointestinal infection in the development of IBS has been confirmed[81]. The final and most promising area is that of alterations in small intestinal microbiota in a subset of patients with IBS[74,82]. Several probiotics[83,84] and antibiotics[85] might play a potential therapeutic role in IBS.

Most studies in this area have investigated the changes in fecal microbiota in patients with IBS[86,87], however, corresponding investigations of the microbial composi-

tion of the small intestine, the duodenum in particular, in patients with IBS are rare[88,89]. A recent specific real-time PCR-based investigation using duodenal mucosa brush samples noted that the percentage of *Bifidobacterium* corresponding to the species *Bifidobacterium catenulatum* was significantly lower in patients with IBS than in healthy subjects[88]. The same group showed higher levels of *Pseudomonas aeruginosa* in the upper small intestine of patients with IBS than in healthy subjects[88]. Although further investigation is required, these findings suggest that therapies involving modulation of the small intestinal microbiota merit consideration. The relationship between SIBO and IBS is highly inconsistent among studies, and there is no evidence of SIBO being absent before IBS symptoms are evident and present after IBS emerges[56].

Duodenal flora and CD

CD typically presents in early childhood with chronic inflammation of the small intestinal mucosa and permanent intolerance to dietary gluten. Several studies have confirmed that other factors such as abnormalities in the small intestinal microbiota might be associated with this disorder[90-93]. Nadal *et al*[91] conducted bacteriological analyses of duodenal biopsy specimens based on pediatric patients with CD. Their results showed that patients with active CD had significantly higher numbers of total bacteria, especially Gram-negative bacteria, compared with asymptomatic patients and healthy subjects[91]. The ratio of *Lactobacillus-Bifidobacterium* to *Bacteroides-Escherichia coli* was lower in patients with CD. Nistal *et al*[92] analyzed the bacterial *16S rRNA* gene sequencing of DNA extracted from duodenal biopsies and showed that the diversity of duodenal microbiota was significantly different between treated and untreated adults with CD due to treatment with a gluten-free diet. Furthermore, Di *et al*[93] found that a gluten-free diet lasting two or more years could not completely restore the microbiota. Undoubtedly, the fecal-associated microbiota composition and related metabolites could also be disturbed in patients with CD[94,95]. Disruption of the duodenal microbiota in patients with CD was linked overall to the symptomatic presentation and could favor the pathogenesis of CD.

The composition of the microbiota within the small intestine has not been analyzed comprehensively using a high-throughput *16S rRNA* gene or metagenomic sequencing method, either in healthy individuals or in patients with gastrointestinal conditions. The study of specimens from the small intestine (especially the distal duodenum, jejunum, and proximal ileum) collected from organ donations and transplantation could be a good way of understanding the abundance and variety of normal microbiota within the small intestine.

STUDY LIMITATIONS AND FUTURE OF HUMAN MICROBIOTA RESEARCH

It is evident that gastrointestinal microbiota contribute to human health and disease. The composition and function of microbiota within the human gastrointestinal tract have been sought for decades, but efforts have been hampered by the following factors: the complexity of gastrointestinal microbiota, especially with regard to the abundance and diversity of commensal fungi and viruses within the human gastrointestinal tract[96,97]; the heterogeneity and multifactorial pathophysiology of gastrointestinal diseases; the impact of the variability of host genotype, diet[98,99], age[100,101], race[98], geographic location[98], drug treatment[102], and medical intervention[103] on gastrointestinal microbiota; inherent limitations in the methodologies used to assess the composition and function of gastrointestinal microbiota; and a lack of suitable animal models (similar to human microecology) for studying the pathogenesis of various disorders. High-throughput sequencing and bioinformatics analyses are evolving rapidly and providing us with fascinating insight into the microbiota present within the human gastrointestinal tract. We are in the midst of a revolutionary period with respect to investigation of the gastrointestinal microbiota. There have been remarkable advances with respect to establishing which microbes are altered in healthy subjects[19] *vs* those in individuals suffering from IBD[1], obesity[1], and type 2 diabetes[23]. However, these studies mainly focused on the fecal-associated microbiota, because the gut microbiota has a major impact on human health and disease and is the best-studied ecosystem; and fecal samples are easy to collect and suitable for the metagenomic sequencing of whole community DNA.

At present, there are more study limitations for the microbiota in the upper gastrointestinal tract, especially the choice of representative human specimens and the application of a reliable analytical method. Endoscopic biopsy specimens, aspirate samples, mucosa brush samples, and surgical specimens from the esophagus, stomach and upper duodenum could be used for microbiota analysis. However, sample collection from the distal duodenum, jejunum, and proximal ileum is still difficult; the surgical and aspirate samples, especially the specimens from organ donations and transplantation may be suitable for analysis. In addition, contamination by the oral microflora and the microbiota from other sections of the upper gastrointestinal tract, and contamination with human host DNA could represent major and permanent methodical problems. Microbiota studies are subject to the restriction of missing distinction between transient and resident microflora in the esophagus, stomach and small intestine, thus, the collection and handling of specimens are of great importance.

A number of culture-based techniques and PCR-based molecular approaches including TRFLP, DGGE and TGGE, RISA, DNA microarray and FISH, have been applied to analyze the human microbiota. Furthermore, the next-generation high-throughout DNA sequencing techniques based on 454 pyrosequencing or Illumina (Solexa) sequencing platforms are the most powerful to investigate the composition, abundance and function of the gastrointestinal microbiota. The analytical method

selected for the assessment of upper gastrointestinal microbiota will depend on the expected target as well as the time and cost-effectiveness associated with the research. Currently, the high-throughput sequencing of 16S rDNA, but not the metagenomics sequencing of the whole microbial community DNA, may be the best molecular method for upper gastrointestinal microbiota. However, it is difficult to distinguish DNA coming from dead or live microbes, when using extracted DNA in the PCR-based molecular analysis. At present, both molecular and culture-based methods should be used to investigate the microbiota composition in the human gastrointestinal tract. Although it could be an arduous task, it is essential for scientific researchers to sequence and characterize the microbiota within the upper gastrointestinal tract. In the future, the use of metagenomics combined with human genome-wide association studies, as well as metabonomics and metaproteomics, may be an ideal approach to understand the microbiota-host interaction and unravel the significance of specific microbiota to determine which microbiota are causative and which are present merely as a consequence of disease. Perhaps one day specific microbes and microbiota-based biomarkers will be developed for diagnostic and therapeutic purposes.

CONCLUSION

In summary, the upper gastrointestinal microbiota is implicated in several gastrointestinal illnesses. There are many study limitations for the upper gastrointestinal microbiota, which could be prevented or mitigated. Through the conceptual innovations in metagenomics and the improvements in DNA high-throughput sequencing and bioinformatics analysis technology, it is now possible to explore the genetic nature of the microbiome in the esophagus, stomach, and small intestine, and the interactions between the host and the residing microbial community.

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