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Swati Sharma
Department of Zoology, DPG Degree College, Gurugram, Haryana, India

Corresponding Author:
Swati Sharma
Department of Zoology, DPG Degree College, Gurugram, Haryana, India

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Exploring the gut microbiome through next-gen sequencing approach

Swati Sharma

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Abstract
The gastrointestinal microbiome is a complex and dynamic ecosystem, made up of hundreds of bacteria, viruses, eukaryotes, and archaea that plays a vital role in maintaining human health. The importance of the gut microbiome in the study of biological microbes has been demonstrated in recent years. The human gut microbiome is considered an essential source of the human bacterial population and has significant contributions to both beneficial and detrimental physiological effects. The study of this intricate community holds the key to the understanding of disease pathogenesis and, thus, for developing novel diagnostic and therapeutic approaches. Technical and methodological variations for microbial extraction and analysis pose various challenges to the study of human microbiome. Microbes and bacteria must be examined in order to identify the causal microorganism. We need new and effective ways to treat diseases. Sequencing can be expanded by using bioinformatics and next-generation sequencing to help analyse vast amounts of sequenced data for bacterial research. However, the scope of bioinformatics identification and analysis has expanded due to breakthroughs in sequencing technology. Next-generation sequencing methods including meta-transcriptomic, 16S rRNA, and metagenomic sequencing have allowed the collection of crucial experimental data about the immune responses generated by the gut microbiota in response to genetic manipulations which appear to be useful in diagnosing illnesses. The biology of microbiome related disease development and treatment is still poorly understood, despite tremendous advancements over the last couple decades. The current study, thus, emphasises on the management of gastrointestinal issues by making use of microbial gene sequencing and information analysis.

Keywords: Metagenomic sequencing, Next-generation sequencing, Human gut microbiome, Sequence analysis

Introduction
The human body is a complex ecosystem, hosting an astonishing array of microorganisms. The human gut microbiome, consists of millions of microorganisms that colonize the human gut. The term "microbiome" refers to a heterogeneous community of various types of microorganisms like bacteria, fungi and viruses that inhabit various organs within the human body, like GIT, oral and nasal cavity. This microbiome often shows a symbiotic relationship with its human host significantly contributing to numerous physiological processes in turn, promoting human health and wellbeing. Microbes are found inside the bodies of humans and animals alike. It has been known to humans for many years that in order to maintain a general well-being, the diversity and balance in the internal microbiome is essential. The gut microbiome is considered as an organ in itself with its unique complexity performing highly specific functions [1].

The microbiota constituting the gastrointestinal tract is able to perform multiple functions for the human body, like nutritional, physiological and immunological functions, which are distinct from the host's own constitutive resources [2]. It has been established that an animal's gur and its brain are interconnected through a gut-brain axis. This axis is crucial in determining not only the gut related physiology but can also influence our dietary choices, psychology and emotions.

Therefore, it is essential to study the structure and function of the gut microbiome in order to understand its role in maintaining the normal physiology and in the development of diseases. Recent research on the gut microbiome has been transformed by next-generation sequencing technologies, which have yielded insights into the makeup, diversity, and function of the microbial communities that inhabit the human gastrointestinal tract, which were previously unheard of [3].

Conventionally, research on the gut microbiome has been limited due to the difficulties in procuring and maintaining cultures of the gut microbial species under laboratory conditions [4]. Advancements in next generation sequencing (NGS) techniques, have enabled researchers to bypass the traditional culture dependent bias and have significantly broadened the understanding of the diversity, composition, and functions of the gut microbiome in human health and disease. These advancements have been proving to be extremely useful in opening new avenues for the development of targeted therapies for gastrointestinal disorders and other related conditions [5].

NGS-based metagenomics enables the isolation and sequencing of all genetic material within a given sample, providing a comprehensive data of the microbiome's genetic makeup [6]. The emergence of metatranscriptomics and metaproteomics, which analyse the expressed genes and proteins of microbial communities, respectively, provides further insights into the activities of uncultivated microbes [7]. The application of NGS technologies in gut microbiome research has led to the discovery of novel microbial species, metabolic pathways, and interactions within the gut ecosystem [8]. The study of microbiome can have numerous clinical applications, such as diagnosis of an infection or a diseased condition, monitoring and control of a disease or infection and identification of mutation patterns, to name a few. The current study discusses the nature of human gut microbiome and how NGS can help in enhancing our knowledge of the microbiome information, in order to help manage human gut issues.

The three generations of sequencing technologies

Nucleic acid sequencing involves the reading of nucleotides in the sample in the correct order of their placement, thereby determining the structure of the nucleic acid. This “reading” of the sequence is important to understand the possible functions of the molecule. DNA sequencing techniques evolved over time and are now widely studied under three generations of sequencing technologies. The first-generation sequencing included the techniques which employed chemical or enzymatic degradation of nucleic acids like, Maxam-Gillbert’s chemical degradation technique and Frederick Sanger’s ddNTP based chain termination-based sequencing method. This technique used different dideoxynucleotides, which were used to terminate the DNA chain elongation during the process of replication. As a result, sequence reads of up to a few hundred nucleotides in length could be obtained. Due to its much more rapid and fairly accurate results, Sanger’s method was widely adopted and thus revolutionized the field of biology [10].

Second generation sequencing made possible high-throughput sequencing through the introduction of massively parallel sequencing, allowing for the simultaneous sequencing of millions of DNA fragments using platforms like Illumina and Ion Torrent. However, one of the pioneers in the second-generation technologies was Roche’s 454 sequencing. This method makes use of pyrosequencing in order to allow the identification of the nucleic acid sequence, by monitoring the released pyrophosphate molecule after the addition of nucleotides to the DNA template [10]. Another platform that determines the nucleic acid sequence is Ion Torrent. It does so by detecting the release of hydrogen ions during the synthesis of DNA molecules. Reversible dye terminators are the basis of the sequencing-by-synthesis technique used by the popular Illumina sequencing platform. Another emerging technology is sequencing of nucleic acids by ligation method, that is, Oligonucleotide Ligation and Detection (SOLiD). This technology uses a ligation-based method with reversible terminators to ascertain the DNA sequence. The speed and throughput of DNA sequencing have been greatly enhanced by these second-generation sequencing technologies, opening up a variety of uses in clinical diagnostics and genomics research [11].

The most recent advances in DNA sequencing are exhibited by third-generation sequencing technologies, that offer novel approaches that go beyond the drawbacks of earlier generations. Compared to prior methods, these technologies are capable of long-read sequencing allowing for the sequencing of significantly larger DNA fragments. One example is PacBio Sequencing, which uses fluorescently tagged nucleotides in a single-molecule, real-time (SMRT) technique to enable long-read sequencing of DNA fragments up to tens of kilobases in length. Another example of third-generation sequencing technique based on nanopore technology is Oxford Nanopore. This technology gives portability, long-read lengths, and real-time analysis, by detecting electrical current variations to ascertain the DNA sequence, as single-stranded DNA molecule travels through a nanopore.

Next-generation sequencing in the study of gut microbiome

In current times, the rapid and efficient analysis of results is indispensable for disease management. The development of NGS technology has made it easier to study the gut microbiome and to investigate the functional and genetic diversity of uncultured gut microbial populations at reasonable costs and with a high enough throughput. NGS can be used to study the identification of microbial species through two major techniques. These are, amplicon profiling through the sequencing of 16S rRNA and shotgun metagenomics. One of the most popular techniques for describing the diversity of the gut microbiome is amplicon-based profiling. Here, PCR is used to target and amp up a taxonomically informative gene marker from the total DNA, often 16S rRNA for bacteria and archaea, which is common for species to be examined. After sequencing the resultant amplicons, subsequent bioinformatics analysis is carried out to ascertain the sample's relative taxonomical abundance. But amplicon sequencing usually only determines the gut microbiome's taxonomic composition. Biological processes linked to the gut microbial community cannot be directly established. Thus, by connecting 16S rRNA gene information with reference genomes, newly developed computational techniques, as those used in PICRUSt [13] and Genome characteristics [14] are successfully used to predict the functional capacity of the community.

The alternative approach to 16S rRNA analysis is shotgun genomic sequencing, used for characterizing the gut microbiome. In this technique, the total DNA is sequenced and examined rather than being amplified against a particular gene marker. A fair and non-speculative method for detecting microorganisms, the mNGS methodology may produce results in as little as 12 to 24 hours. Additionally, it detects drug resistance genes, separates viral, infectious, and parasitic life forms, and reduces the difficulties associated with generating critical organic entities. The anticipated benefits of mNGS over culture and other approaches assure that it is better for clinical applications, adding to the observation that advancements in mNGS innovation and information study have reduced the expense of testing.

As per known literature, the human microbiome project has successfully completed a vast gene catalog that includes over 9.8 million microbial genes. The varied nature and variety of the human microbiome are outlined in this catalog in a progressive manner. The information comes from the examination of metagenomic sequencing data from 1,267 gut metagenome samples that were taken from 1,070 people. 760 samples from the Meta HIT project in Europe, 139 samples from the Human Microbiome Project in the US, and 368 samples from a sizable diabetic group study in China are among the samples from different demographic groups that are included in the sample pool. About 750,000 genes were found in each sample, which is more than 30 times more than there are in the human genome. Notably, more over half of the subjects had fewer than 300,000 genes. Notably, the majority of the rare genes found in this study are found in fewer than 1% of people [15, 16]. These studies hint at the significance of metagenomic sequencing in understanding the complexities of microbial communities and their interactions with the humans.

Our understanding of the gut microbiome has significantly increased due to NGS-based sequencing, yet current culture-independent metagenomics produce heterogeneous data that reflects community-level traits rather than species-specific traits. Consequently, high-throughput culture techniques, typically referred to as Culturomics, are gaining traction again [17]. Single cell genomics has been developed to study uncultivated species from a variety of environments for unculturable microorganisms. The identification of novel species without prior cultivation could be accelerated by single cell genomics, which requires a step for amplifying the genome from a single cell [17].

Understanding the human gut microbiome-basic structure and function

A diverse range of microorganisms, including bacteria, fungi, eukaryotes, viruses, and archaea, are found in the human the gastrointestinal tract. The use of probiotics, antibiotics, laxatives, and prokinetics, as well as environmental exposures and dietary factors, all affect this microbial diversity. The complex interactions within the gut microbiota are essential for maintaining a healthy body through the maintenance of homeostasis, including optimal metabolism, and immunity. High throughput DNA sequencing techniques have revealed that the human gut microbiota comprises of millions of microbial species and communities, most abundant being, *Actinobacteria*, *Bacteroidetes*, and *Firmicutes*. Common bacterial taxa include *Clostridia, Erysipelotrichales, Lactobacillales* (*Firmicutes*) and *Coriobacteriales* (*Actinobacteria*) [9].

Individual differences may exist in the relative distribution of microbes within the same person. Age and environmental factors, such as medication use, can affect a person's gut microbiota. Furthermore, the gut microbiota differs in the GI tract's various anatomical sections. For instance, the small intestine contains *Protobacteria* like *Enterobacteriaceae*, while the colon does not. Rather, the colon is frequently home to *Bacteriodetes* such *Rikenellaceae*, *Prevotellaceae*, and *Bacteroidaceae*. These differences are mostly caused by the differences in various environments and micro-environments [18]. The gut microbiota varies by age in addition to its geographic distribution. In general, the diversity of microbiota rises between childhood and adulthood and falls as people age (beyond 70) [19].

Prior to the development of a comparatively stable gut microbiota composition, *Akkermansia muciniphila*, *Bacteroides, Veillonella*, *Clostridium coccoides* spp., and Clostridium botulinum spp. dominate the variety of children's microbiota [20]. Around age three, the gut microbiota of children resembles that of adults, with the three principal microbial phyla *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* becoming more prevalent. The composition of the human gut microbiota may then be impacted by changes in the immune system and diet as people age. In particular, older adults typically have higher levels of *Clostridium* and *Proteobacteria* and lower levels of *Bifidobacterium* [21]. Because of its function in immune system stimulation, the decline in the anaerobic bacteria *Bifidobacterium* is thought to be related to worsened inflammatory condition.

Gut microbiota composition can largely be affected by the use of antibiotics. Firmicutes and Bacteroidetes become unbalanced as a result of broad-spectrum antibiotics. During the treatments, both the diversity and abundance of these microorganism’s decline. The antibiotic class, dosage, exposure duration, pharmacological activity, and target bacteria all affect how the composition of the microbiome changes [22]. Changes in bacterial composition after antibiotic therapy are partly caused by specific antibiotic features, such as their antimicrobial activity or mode of action, which are strong factors for intestinal microbiota selection. Variations in the physiological gut microbiota have a significant impact on both intestinal and extra-intestinal illnesses. In fact, dysbiosis is frequently described as a change in the composition of the gut microbiota and either the cause or an effect of illnesses. Determining whether a change is good or damaging is frequently challenging [23].

The gut virome is a broad category of DNA and RNA viruses that infect human primary cells, eukaryotic cells, and prokaryotic cells. These viruses can be single-stranded or double-stranded. Virome infections are mainly spread by contaminated food, wastewater, and the oral-fecal pathway. The microflora composition of the human gut microbiome is greatly influenced by pathogens found in the intestinal viromes. The human fecal virome has about 189,680 species, according to metagenomic studies, despite the fact that bacterial components have been studied in greater detail. The comparatively low concentration of eukaryotic viruses in the gut makes it difficult to distinguish between diseases in humans that are caused by bacteria, viruses, or eukaryotes [9].

Implications of Gut Microbiome Modeling in Healthcare and Medicine

The nature of diet and dietary intake is one of the primary factors influencing the makeup of the gut microbiota and subsequently the host metabolism, is diet [24]. The primary macronutrients carbohydrates, proteins, and fats have a significant influence on the composition of the gut microbiota and host metabolism depending on their quantity, kind, and balance. While many dietary polysaccharides, including resistant starch, non-starch polysaccharides, and plant fibers, can be broken down by gut microorganisms but not by the human host, monosaccharides, such as glucose and galactose, are readily absorbed by intestinal epithelium cells. The notion that *B. thetaiotaomicron* is effective at using dietary polysaccharides is supported by the fact that the high-carb diet gives it a rich carbon source and maximizes its growth. Given that *Bacteroides* are known to be incapable of using proteins as their only carbon source and that they have a low capacity for proteolysis, the high-protein diet does not promote the growth of *B. thetaiotaomicron* [25]. Individuals following a high-protein/low-carb diet also showed decreased abundance of *Bacteroides* in their gut [26]. Therefore, based on the nutrient composition of the food, a diet that optimizes the growth of the microbiota and improves human metabolism can be designed to modify the gut microbiota. It has been shown that altering the gut microbiota using probiotics or prebiotics has an impact on the host's metabolism, namely glucose homeostasis [27]. Lactobacillus strains used in probiotic treatment have been well studied for their possible antibacterial properties against common enteric and stomach infections [28]. Therefore, probiotics and their byproducts, known as postbiotics, have been suggested as dietary supplements for improved intestinal homeostasis and as therapeutic tools for the management of IBD [29].

Despite the significant advantage of NGS-based metagenomics in genetic characterization and identification of species diversity, composition and richness, the elucidation of metabolic modifications paralleled with alterations in the metabolite levels remains a big challenge. Since the microbiota is a complex community, the characterization of each and every member along with its exact biological role is extremely difficult. Even with high throughput NGS technologies, the elucidation of the most accurate biochemical pathways is a challenge, since the by-products of genes and genomes are largely affected by environment, epigenetic mechanisms and interspecific interactions of the microbiome constituents. The capacity of GEM-based modeling to foresee microbial metabolism makes it an appealing answer to this problem. Future probiotic strains can be better designed and optimized with increased postbiotic production through metabolic engineering by using GEMs to thoroughly investigate the biosynthesis of active postbiotics. Additionally, the gut microbiota's diverse distribution throughout the gastrointestinal system will result in varying prebiotic actions at various gastrointestinal sites [30].

Challenges and future prospects in NGS based microbiome studies

As NGS technology develops, so does the volume of data that is routinely gathered during each run. However, one of the major hindrances to research in NGS is still bioinformatics based analysis. A bioinformatics core and a qualified bioinformatician are not available to all scientists. Additionally, a large portion of NGS analysis software is designed to function in a Unix/Linux environment [31]. Furthermore, mNGS can identify the whole range of microorganisms, including bacteria, viruses, fungus, and parasites, whereas 16S/18S sequencing is restricted to identifying bacterial and eukaryotic species, leaving viruses out of the research. In comparison with targeted PCR techniques, mNGS is primarily non-biased making this property one of its major advantages.

When employing molecular assays to diagnose polymicrobial illnesses, universal primers provide a problem. Multiple base calls are produced for every nucleotide during 16S sequencing of polymicrobial populations, which leads to an unclear nucleotide chromatogram. Many labs use next-generation sequencing technologies for polymicrobial samples, even though computational techniques can help decode these sequence. Compared to traditional methods, mNGS may be able to detect polymicrobial illnesses and new species more successfully. Even while mNGS has the potential to completely transform microbiological diagnostics, its therapeutic value in modern medicine is yet unknown and needs more research. The clinical relevance of mNGS testing should become more apparent as sequencing and bioinformatics skills develop, opening the door to future treatments that are more individualized and successful [32].

By quantitatively integrating transcriptomics, proteomics, and metabolomics data with metabolic phenotypes, a GEM-based modeling technique aids in the systematic exploration of the gut microbiota. Lastly, to theoretically explain reported metabolic abnormalities, GEMs based on in silico growth or metabolite production prediction can be readily contrasted with collected experimental data. Cross-feeding microbial communities for industry have been successfully developed using GEMs [33]. Through the exchange of cross-feeding metabolites between species, syntrophic growth can be accomplished in these designed microbial communities. Likewise, the syntrophic design approach can be used to create probiotics that are tailored to have improved nutritional catabolism or postbiotic biosynthesis. Furthermore, in order to test and validate in silico models, the modeling technique necessitates the systematic alteration of gut microbiota through well planned *in vitro/in vivo* research. The human intestine microbial ecosystem simulator is one instance of such an *in vitro* system [34]. Lastly, metabolic modeling in conjunction with experimental data and information will significantly improve our comprehension of metabolic interactions between bacteria or between the germ and host, hence offering insight into the clinical applications of gut microbiota in the diagnosis and treatment of various physiological disorders.

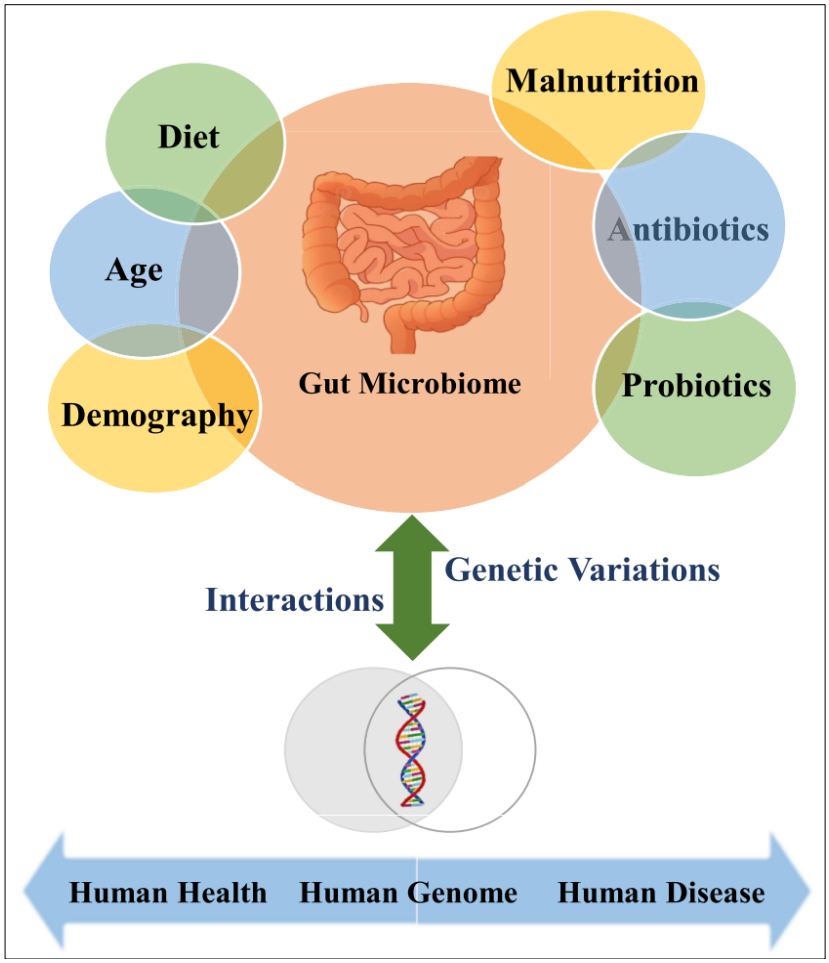

Fig 1: Human gut microbiome and associated factors [9]

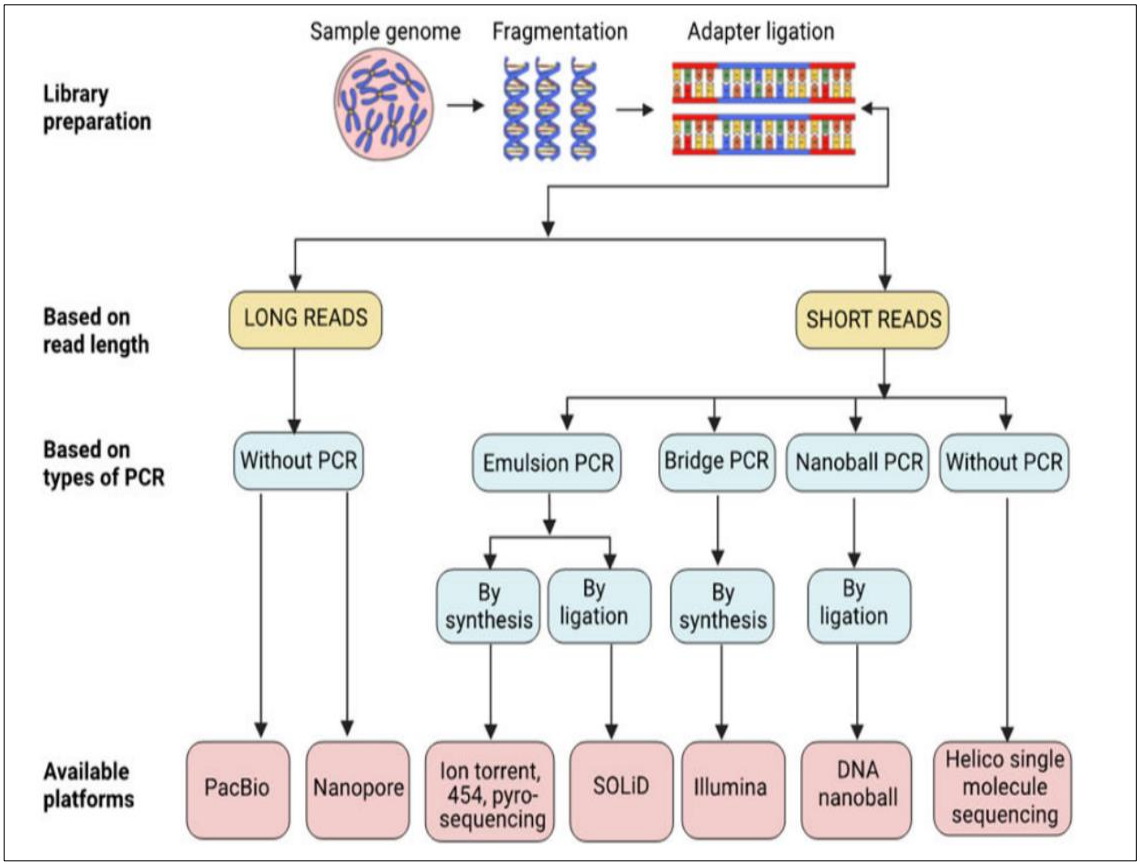

Fig 2: Overview of various NGS technologies with different platforms and principles [12].

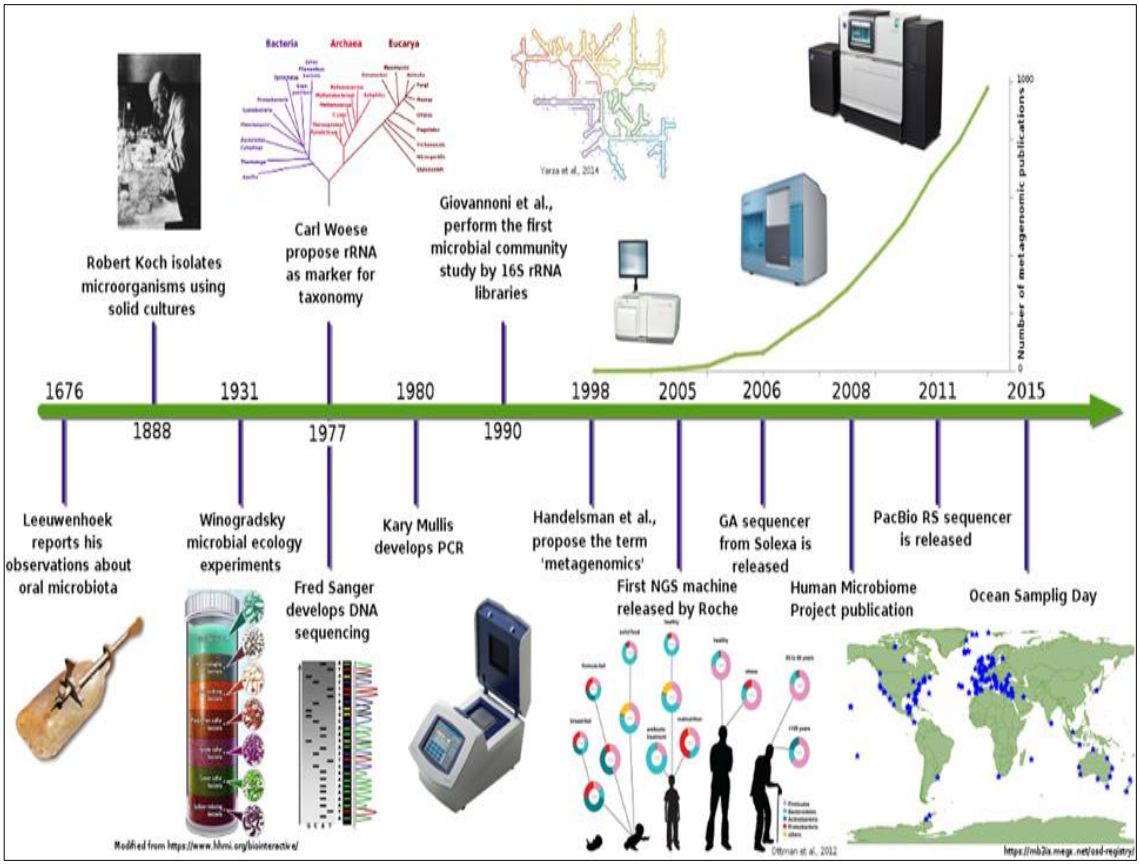

Fig 3: Metagenomics timeline and milestones, timeline showing advances in microbial community studies [6]

Table 1: Comparison of some important parameters between 16S (amplicon) sequencing and shotgun sequencing

	16S/18S/ITS sequencing	Shotgun sequencing
Input	DNA coding for the 16S, 18S ribosomal subunit or ITS	Host and microbial DNA
Recommended sample type	All	Human microbiome
Bacterial/fungal coverage	High	Limited
Cross-domain coverage	No	Yes
False positive	Low risk	High risk
Taxonomy resolution	Genus-species	Species-strains
Host DNA interference	Very limited	Yes, but can be mitigated
Minimum DNA input	10 copies of 16S	As low as 100fg
Functional profiling	No	Yes
Resistome & virulence profiling	No	Yes

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