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Metagenomics as a Diagnostic Method for Identifying Microbiota Profiles in Diarrhea Cases

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ABSTRACT

Diarrhea remains a major global health issue and is one of the leading causes of morbidity and mortality, especially in children. Changes in the composition of gut microbiota are strongly associated with the occurrence of diarrhea, particularly due to infections caused by pathogenic microorganisms. Conventional diagnostic techniques, such as culture, still have limitations in detecting diverse and unculturable microbes. Metagenomics offers a comprehensive diagnostic solution by analyzing the entire genetic material directly from stool samples using sequencing-based approaches. This review discusses two metagenomic strategies—16S rRNA sequencing and whole-genome shotgun metagenomic sequencing—based on studies conducted in India and Peru. The 16S rRNA approach enables the identification of bacterial community structure and dysbiosis in diarrhea cases, while shotgun metagenomics provides higher resolution for detecting pathogenic species, including Campylobacter spp., and co-infections that are often missed by standard methods. Both techniques demonstrate significant advantages in detecting microbiota diversity, identifying novel pathogens, and uncovering antimicrobial resistance profiles. Therefore, metagenomics represents a promising diagnostic tool for improving the accuracy of diarrhea detection and supporting public health interventions in endemic regions.

KEYWORDS *Metagenomics; 16S rRNA; Shotgun Sequencing; Gut Microbiota; Diarrhea Diagnosis*

INTRODUCTION

Diarrhea represents a critical global health burden, ranking among the top three leading causes of death worldwide, with an estimated 1.7 billion cases annually according to the World Health Organization (WHO). This disease disproportionately affects children under five years of age, accounting for approximately 525,000 deaths per year in this vulnerable population. The socioeconomic impact is particularly severe in low- and middle-income countries, where inadequate sanitation infrastructure, limited access to clean water, and poor hygiene practices create endemic conditions. Geographically, the highest burden is concentrated in South Asia and sub-Saharan Africa, where diarrheal diseases contribute significantly to childhood morbidity and stunted growth.

Diarrhea is the third leading cause of death in the world every year due to diarrheal infection. (Organization, 2017) Diarrhea is one of the main health problems faced by Indonesia. Based on data from the Ministry of Health of the Republic of Indonesia, diarrheal disease remains a major cause of high morbidity and mortality rates, especially among toddlers. Some of the contributory factors include poor sanitation, insufficient access to clean water, and low awareness of the importance of hygiene in daily life. Diarrhea can cause dehydration and salt imbalance, both of which play crucial roles in maintaining body homeostasis. One of the most frequent causes of diarrheal illness is bacterial infection. Diarrhea occurs in the intestinal tract and may be caused by various bacterial, viral, and parasitic organisms. This infection can result from consuming contaminated food or water or from poor sanitation (Indonesia, 2021).

The gut microbiota in healthy individuals consists of several microorganisms, including bacteria, viruses, and others that play important roles in digestion, vitamin synthesis, protection against pathogens, and regulation of the immune system. Microbes maintain internal balance and interact intricately with one another. An imbalance in these microbes can lead to intestinal infection and subsequent diarrhea (Chen et al., 2018; Odamaki et al., 2016).

In individuals experiencing diarrhea, the gut microbiota profile can change significantly compared to that of healthy individuals. In such cases, pathogens such as enteropathogenic Escherichia coli, Salmonella, Shigella, and Clostridium difficile, as well as viruses such as rotavirus and norovirus, often increase. Additionally, dysbiosis can lead to a reduction in healthy commensal bacteria, such as Bifidobacterium and Lactobacillus, which help protect against infection and support gut health (Handelsman, 2004).

In individuals who experience diarrhea, the profile of the microbiota in the gut can change significantly compared to healthy individuals, a phenomenon termed dysbiosis. This microbial imbalance triggers diarrhea through several specific mechanisms. First, dysbiosis disrupts the integrity of the intestinal barrier by reducing the production of short-chain fatty acids (SCFAs) and mucin, which normally protect the gut epithelium. Second, pathogenic bacteria compete with beneficial commensals for nutrients and adhesion sites, allowing opportunistic pathogens such as enteropathogenic Escherichia coli, Salmonella, Shigella, and Clostridium difficile to proliferate. Third, the altered microbiota composition stimulates excessive inflammatory responses through the activation of pro-inflammatory cytokines, leading to increased intestinal permeability and secretory diarrhea. Additionally, dysbiosis results in a decrease in the number of beneficial commensal bacteria, such as Bifidobacterium and Lactobacillus, which help protect against infection and support gut health. Understanding these specific microbiota profiles is therefore essential for accurate diagnosis, as it enables clinicians to identify the underlying microbial disturbances driving the disease and to tailor targeted therapeutic interventions.

Previous research has demonstrated the potential of metagenomics in diarrhea diagnosis, though significant gaps remain (Norman et al., 2014; Patel et al., 2012). Pop et al. (2014) pioneered the use of whole-genome shotgun sequencing to identify enteric pathogens directly from stool samples, revealing that metagenomic approaches could detect pathogens missed by conventional culture in up to 40% of cases. Zhou et al. (2019) applied 16S rRNA sequencing to characterize gut dysbiosis in pediatric diarrhea patients in China, identifying distinct microbial signatures associated with different etiologies. More recently, De et al. (2020) combined 16S rRNA and shotgun sequencing to analyze the microbiome and resistome of diarrheal patients in India, uncovering extensive antibiotic resistance genes and environmental microbes not typically detected in clinical samples. (Janda & Abbott, 2007; Miller et al., 2013) utilized shotgun metagenomics to identify complex Campylobacter co-infections in Peruvian children, demonstrating that PCR-based methods miss approximately 65% of actual infections. Despite these advances, most studies have been conducted in isolation, focusing either on bacterial community structure or pathogen identification, but rarely integrating both approaches systematically. Furthermore, there is limited synthesis of findings across different geographic and socioeconomic contexts, particularly comparing low- and middle-income countries where the burden of diarrheal disease is highest.

In identifying the pathogens that cause diarrhea, various methods are used, such as culture. However, this method has limitations in terms of sensitivity and the inability to detect diverse or unculturable microorganisms. Metagenomics is one of the approaches used to analyze all genetic material in a given environment, such as soil, water, or even human organs. Through this method, it is possible to identify all microorganisms in the intestine—including bacteria, viruses, fungi, and parasites—without isolation or culture. With metagenomics, DNA can be analyzed directly from samples. The use of NGS (Next-Generation Sequencing) techniques allows for the identification of microorganisms that may not be detected through culture methods (Wenzl, 2012).

The novelty of this literature review lies in its systematic comparison between 16S rRNA sequencing and whole-genome shotgun sequencing, specifically in the context of diarrheal disease diagnosis, synthesizing evidence from two distinct epidemiological settings—India and Peru. Unlike previous reviews that focus on general methodological aspects, this work critically evaluates the complementary strengths and limitations of both approaches in detecting dysbiosis, identifying pathogenic species, uncovering co-infections, and profiling antimicrobial resistance. Furthermore, this review emphasizes the translational potential of metagenomic findings for clinical practice and public health surveillance in resource-limited settings, providing a framework for integrating metagenomic technologies into routine diagnostic workflows in low- and middle-income countries where diarrheal diseases remain endemic.

The high incidence of diarrheal diseases in Indonesia suggests that applying metagenomics could serve as an effective diagnostic approach. With more precise and accurate identification of the gut microbiota profile in individuals affected by diarrhea, metagenomics can also help detect new pathogens or pathogenic variants that may be responsible for diarrhea but remain undetectable through culture or conventional methods (Keusch et al., 2006).

METHOD

Metagenomik 16S rRNA

In a study conducted by Rituparma et al. on diarrheal patients in Kolkata, India, the focus was on analyzing the gut microbiome and resistome from 20 patient samples collected. Five of the 20 samples were analyzed for resistomes to identify signs of Antimicrobial Resistance Genes (ARGs). The researchers used the 16S rRNA amplicon sequencing method to identify the structure of bacterial communities, followed by analysis using Whole Genome Sequencing (WGS) on five samples (Chakravorty et al., 2007; Quince et al., 2017; Schloss & Handelsman, 2004). This analysis was performed to identify antimicrobial resistance genes as well as diarrhea-causing microorganisms such as Vibrio cholerae, Helicobacter pylori, and other pathogens (Walker & Black, 2010).

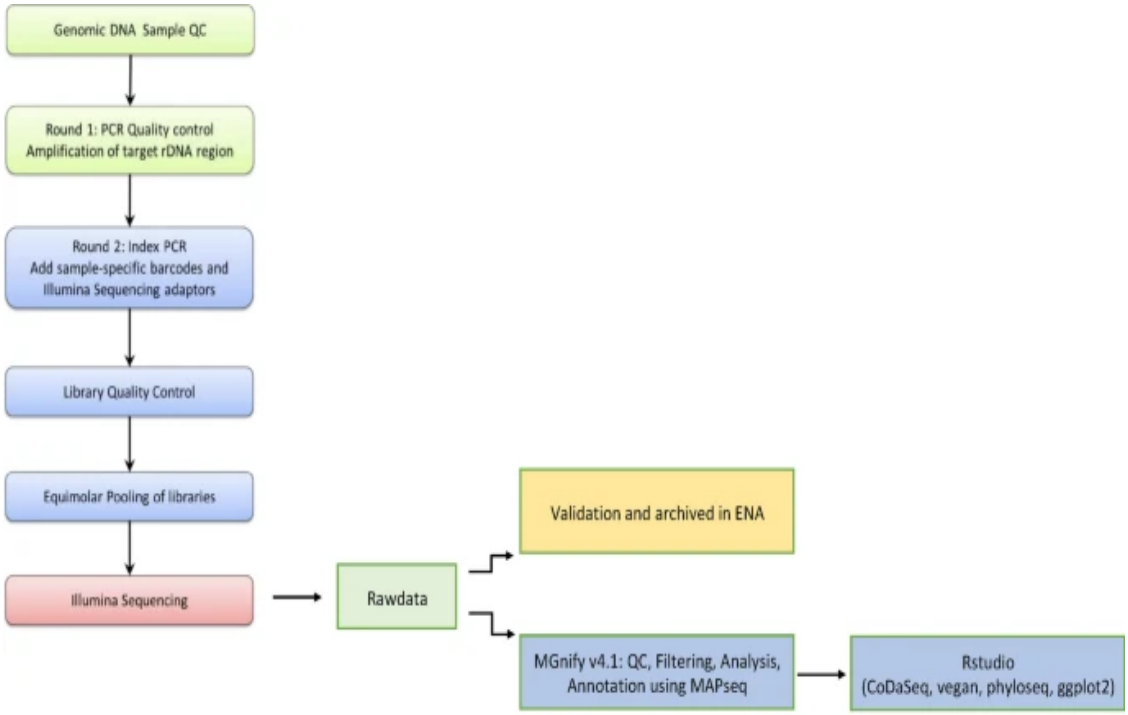


Figure 1. Amplicon 16S rRNA19 Sequencing Process Flow Diagram

In this study, the method used to study the composition of the intestinal microbiota of diarrheal patients using the 16S rRNA technique

1. Sampling

The samples used were feces from diarrhea patients as many as 20 fecal samples from hospitals in Kolkata, India, both from outpatient and inpatient patients.

2. DNA Extraction

The samples were then extracted using a DNA extraction kit that is specific to the fecal microbiota. This DNA is then used for the amplification of the 16S rRNA gene.

3. Gene 16S rRNA Amplification

Amplification is done to obtain gene fragments that represent different types of bacteria. The 16S rRNA gene is used as part of the highly conservative bacterial DNA and is often used for bacterial identification. Certain parts of this gene are usually in the V3-V4 region, then amplified using a universal primer specific to that gene.

4. Sekuensing (Illumina MiSeq Platform)

The results of the amplification of the 16S rRNA gene were then sequenced using a Next-Generation Sequencing (NGS) sequencing platform, such as Illumina MiSeq. This method will later produce millions of "reads" representing various bacterial species in the sample in the form of Rawdata.

5. Mgnify Pipeline Data Processing

The sequencing data obtained is then analyzed using the MGnify pipeline. This pipeline will process the raw data generated from the sequencing and then filter from quality, and align it with taxonomic databases such as the Genome Taxonomy Database (GTDB-Tk) for the identification of bacteria down to the species level.

Shotgun Metagenomic Sequencing

In a study conducted by Craig et al., on fecal patients in children in Peru analyzed to identify species of Campylobacter that play a role in diarrheal diseases experienced by children. The fecal sample used was a sample of children under 2 years old, who were diagnosed with acute or asymptomatic diarrhea. In this study, a positive fecal sample of Campylobacter species was identified using a PCR probe based on the 16S rRNA gene, then it was further differentiated for C. jejuni and C. coli types using qPCR using the cadF gene. It further used the Whole-Genome Shotgun Metagenomic Sequencing (WSMS) method as CIDT to detect and identify the presence of Campylobacter species other than C. jejuni and C. coli in fecal samples from children in Peru (De et al., 2020; Heyman, 2006; Parker et al., 2022; Sharpton, 2014).

1. Sample Collection

Fecal samples used in this study were collected from children in Peru who had previously tested positive for Campylobacter by PCR, but were negative for the cadF genes associated with C. jejuni and C. coli. The sample is then stored at a temperature of -80°C before further analysis.

2. DNA Extraction

DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit where each extraction set used a negative control to detect contamination.

3. Sekuensing DNA

After DNA was extracted with Illumina sequencing using the KAPA High-Throughput Library Preparation Kit with Standard PCR Amplification Module (Kapa Biosystems, Wilmington, MA) on fecal samples using the kit, with an average reads length of more than 188 nucleotides. At this stage, the various species of microorganisms present in the sample have not been identified.

4. Identification of Campylobacter species with the Shotgun Method of Metagenomics

a) Identify Campylobacter species using the shotgun metagenomics method by mapping to the Campylobacter reference genome using Geneious Prime software. In this process more than 28 Campylobacter genomes were used as references, including species C. jejuni, C. coli, C. infans, and others.
b) A total of 44 samples from shotgun metagenomics were obtained with reads ranging from 1,404 to 2,975,084 which were then mapped with the Map to Reference Command to map reads to the relevant genome, with low sensitivity (<10% mismatch between reads and reference genomes).
c) Results that correspond to conservative regions, such as rRNA genes, that can be found in Campylobacter species or other genera of bacteria, will be removed so as not to give false positive results. These results are called non-confirmatory reads, because there is a match for more than one species.
d) After the grouping, the process continues with the identification of the specific Campylobacter species. Results confirmed as species-specific will be verified using BLASTn to compare with the NCBI database and ensure that the results are unique to one Campylobacter species only.

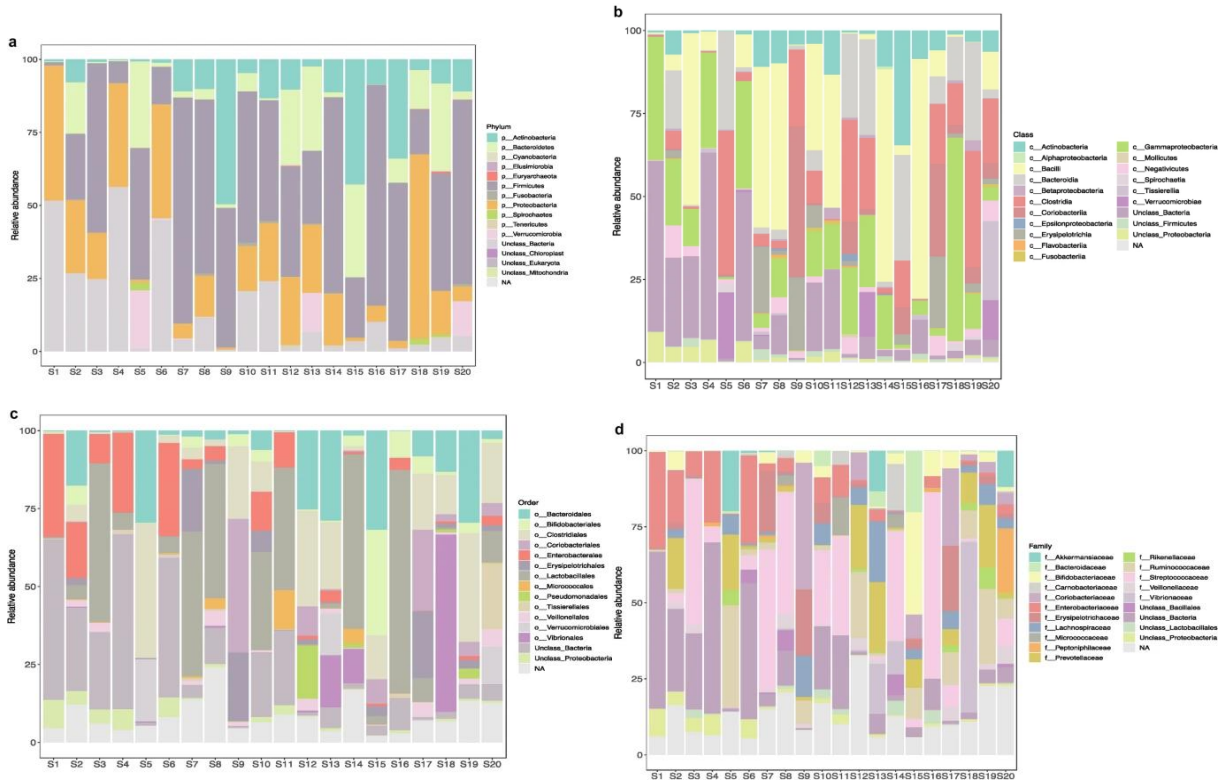
e) The species will be detected if at least one result is confirmed with the Campylobacter-specific genome. If more than 50 results map to a single genome, all reads are tested with BLAST to determine if they are truly unique to the species.

In Shotgun Metagenomics' data analysis, in addition to using Geneious Prime for mapping, the MetaWRAP pipeline can also be used to characterize the overall microbial population in the sample. This pipeline helps in characterizing the entire population of microbial samples from the sample, with the following steps:

a. Trim Galore (v0.6.5) is used to cut and clean sequence reads to improve data quality.
b. The cleaned reads were then mapped with human genome references using BMTagger to remove human DNA contamination on GRCh37/hg19 references.
c. Next, it uses metaSPAdes (v3.12.0) to reassemble the reads into contigus, which will then be assessed using QUAST, then the taxonomic results of the constructs will be assembled at the beginning and all the sequence reads results will be determined using Kraken2 (v2.0.8) against the standard Kraken2 database and visualized using KronaTools.
d. It was further analyzed using the Metabat2 and CONCOCT programs to classify the contiguous based on genetic similarity.
e. Then by using CheckM (v1.02.12) to verify the completeness and contamination of the grouping results.

RESULT AND DISCUSSION

Metagenomik 16S rRNA



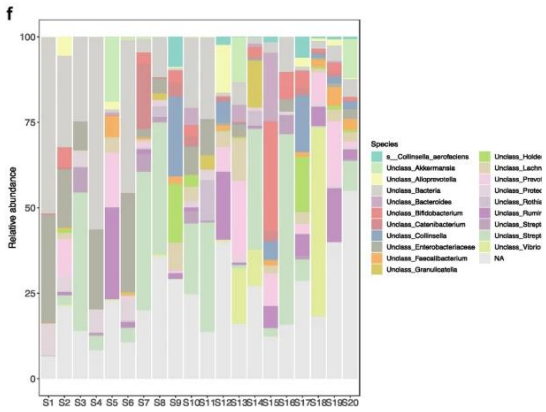


Figure 2. Histogram showing a. Phylum, b. Class, c. Order, d. Family, e. Genus, f. Species19

A total of 46 bacterial phylum were found through DNA sequence homology with the Genome Taxonomy Database (GTDB-Tk). In 20 samples, there were phylums of bacteria, namely Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria.

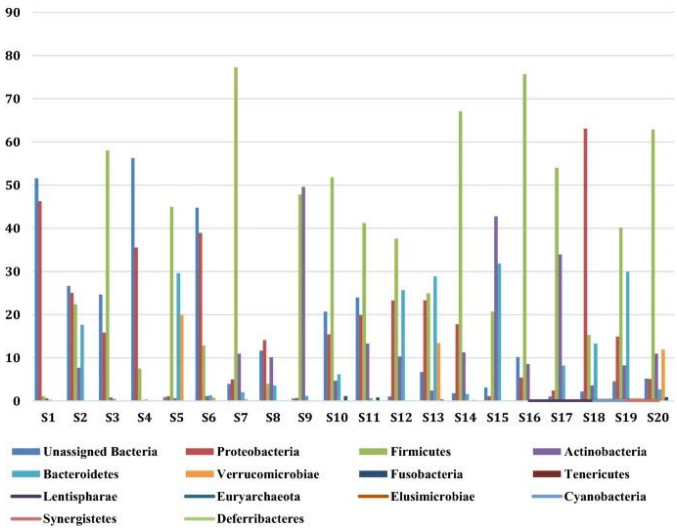


Figure 3. Percentage of Bacterial Phylum in 20 Samples19

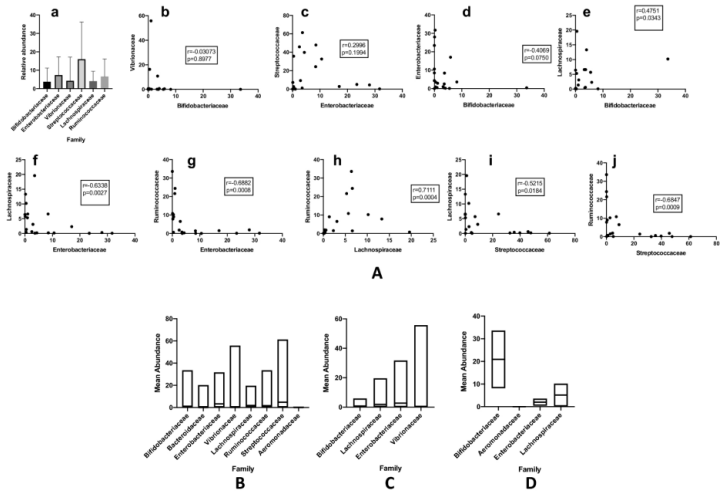


Figure 4. Comparison of Bacterial Families19

After identification, it is then analyzed to see the composition of the bacterial community in each sample. Looking at phylum, classes, orders, and genus are analyzed and expressed in the form of percentages. In this study, it was found that Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria were the phylum most commonly found in diarrhea samples.

In Figure 4, a comparative analysis of the number of bacterial families in diarrhea samples was conducted. This diagram shows some correlation analysis and comparisons between commensal and pathogen families (Field, 2003).

a. Panel A (a-j) shows the Spearman correlation between different families of bacteria such as Bifidobacteriaceae, Enterobacteriaceae, Vibrionaceae, Streptococcaceae, Lachnospiraceae, and Ruminococcaceae.
b. A significant positive correlation was seen between Lachnospiraceae and Ruminococcaceae, suggesting that if one family of bacteria increases, others also tend to increase.
c. Significant negative correlations were found between Enterobacteriaceae and Ruminococcaceae, as well as between Enterobacteriaceae and Lachnospiraceae, indicating that an increase in one family is often followed by a decrease in the other.
d. Panel B-D shows the relative abundance ratio between bacterial families in samples associated with pathogens such as Vibrionaceae and Aeromonadaceae
e. The abundance of the pathogenic bacterial family compared to the commensal family showed a pattern that varied among the samples, but was not statistically significant.
f.

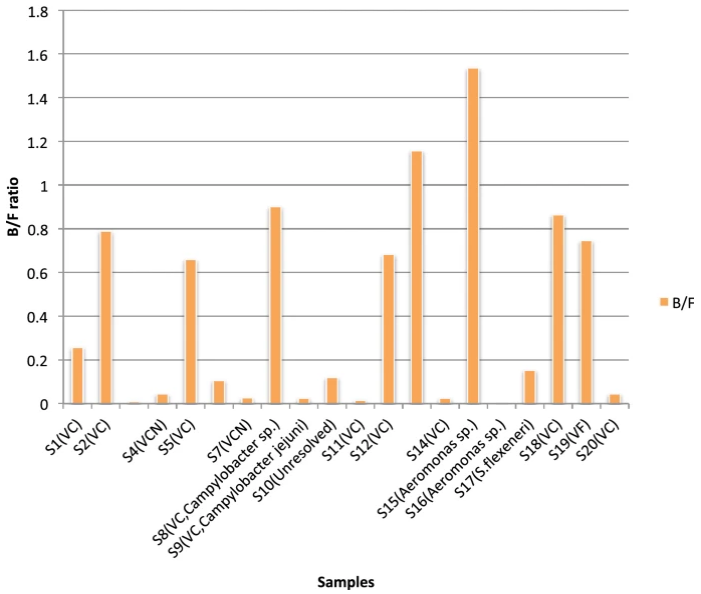


Figure 5. Ratio of Bacteroidetes/Firmicutes (B/F) in Diarrheal Samples19

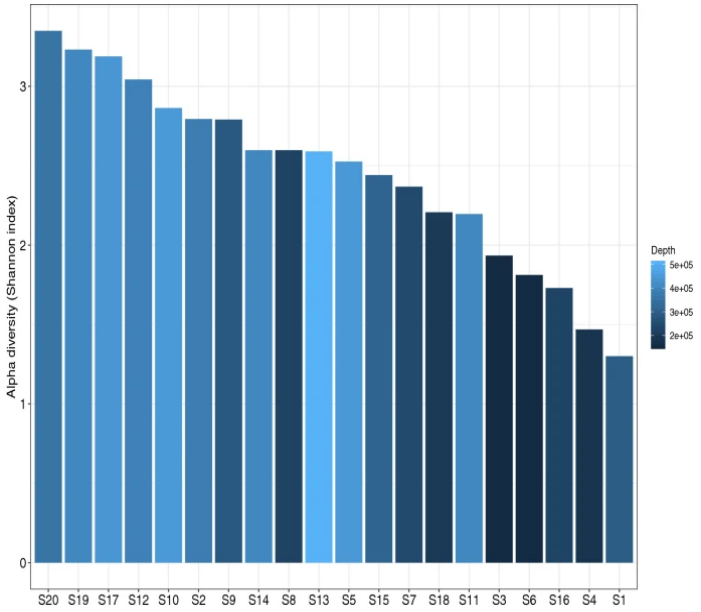


Figure 6. α-Diversity of Twenty Samples of Diarrhoeal Patients19

This ratio is an important indicator for predicting dysbiosis or an imbalance of the gut microbiota. The results showed that the B/F ratio was lower than 1 in almost all samples, except in the S13 and S15 samples, where Bacteroidetes were more dominant than Firmicutes.

The diarrhoeal pathogens isolated from each sample are indicated in parentheses next to the sample code. This provides context on how specific pathogens may affect the composition of the microbiota, including the observed B/F ratio.

Figure 6 above explains the α-diversity of twenty diarrhea samples measured using the Shannon index, which measures the richness and evenness of the microbiota in each sample.

a. Sample S20 has the highest α-diversity, indicating that it has the most number of microbes and a more even distribution of microbes.
b. In the S1 sample, it has the lowest α-diversity, indicating that the microbiota in this sample is not only few but also less even.
c. Although some samples contained the same diarrhoeal pathogen, such as Vibrio cholerae O1 in S1 and S20, the two samples showed large differences in α-diversity. This suggests that other factors, such as environmental conditions or individual-specific factors, may have an effect on the structure of the patient's gut microbiota.
d. Several samples isolated from different diarrhoeal pathogens, such as S8, S13, and S14, showed the same Shannon index, indicating that despite the presence of different pathogens, the microbial richness and balance in these samples were nearly identical.

5. Interpretasi

The results of the 16S rRNA sequencing were interpreted to understand the composition of the microbiota. This analysis is also used to look at the relationship between the presence of commensal bacteria and pathogens, as well as to identify changes in the composition of the microbiota that may be associated with diseases such as diarrhea.

A total of 584 genera were observed, 136 of which could be further classified down to the species level, while the remaining 448 could not be further classified by sequencing 16S rRNA amplicon.

Shotgun Meganenomic Sequencing

Of the 50 fecal samples examined and analyzed, they successfully sequenced and obtained data from 44 samples. The total DNA recovery of 44 samples varied quite a bit and resulted in total reads per sample between 1,410 and 2,975,084 paired-reads, while overall 84% of the sample had more than 150,000 reads.

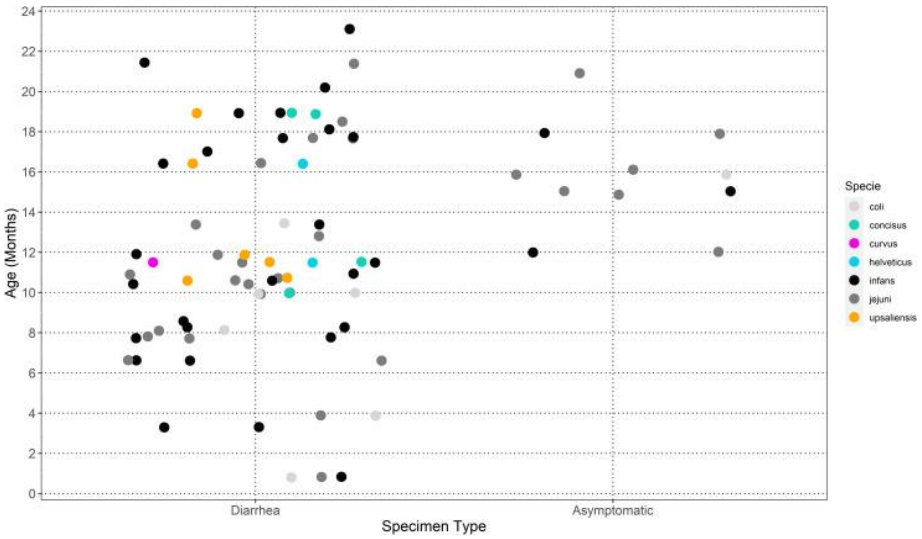


Figure 7. Campylobacter Species in Symptomatic and Asymptomatic Fecal Samples20

In the image, various species of Campylobacter were found in fecal samples from children with diarrhea and asymptomatic children. This figure shows the distribution of species such as Campylobacter concisus, C. curvus, C. helveticus, and C. upsaliensis, which were found mainly in samples from children with diarrhea, especially in children over 10 months of age.

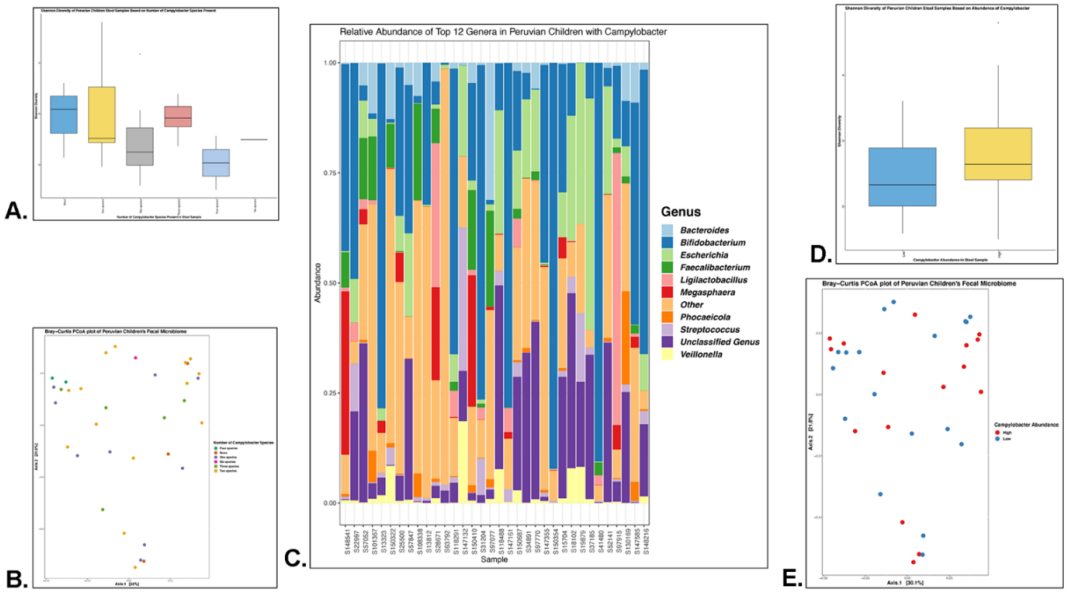


Figure 8. Analysis of the Fecal Microbiome Used in Research from the Shotgun Process of Metagenomics20

a. Shannon Diversity shows microbiome diversity based on the number of Campylobacter species in fecal samples. However, the results of the analysis showed that there was no significant difference (p-value = 0.24) between samples with different numbers of Campylobacter species.
b. The Bray-Curtis PCoA Plot shows the diversity of the microbiome (beta diversity) using the Bray-Curtis Principal Coordinates Analysis (PCoA) method. The plot compares the diversity of microbiome communities based on the number of Campylobacter species. Just as in Shannon's analysis, no significant differences were found (R^2 = 0.1948, p-value = 0.273).
c. The Taxonomic Barplot shows the relative abundance of the top 12 microbial genes found in fecal samples from the children involved in the study. A total of 34 of the 44 samples are shown in this graph, while the other 10 samples did not have enough sequence reads to be included in the analysis.
d. This Shannon Diversity by Campylobacter abundance graph evaluates Shannon diversity based on the relative abundance of Campylobacter in fecal samples. The results also showed no significant difference (p-value = 0.733), suggesting that the amount of Campylobacter present in the sample did not affect the overall microbiome diversity.
e. The Bray-Curtis PCoA plot based on Campylobacter abundance shows a relationship between gut microbiome diversity and Campylobacter abundance in samples. The results of the analysis showed no significant difference (R^2 = 0.0653, p-value = 0.149)

Whole-Genome Shotgun Metagenomic Sequencing (WSMS) is able to efficiently identify Campylobacter species other than C. jejuni and C. coli. Other non-jejuni and non-coli Campylobacter species can be found among children in Peru, among them Candidatus "C. infans" which is the most commonly identified Campylobacter species, although C. upsaliensis, C. concisus, C. helveticus, and C. curvus are also identified.

The study used shotgun metagenomics techniques to analyze Campylobacter infections in children in Peru, where it was found that more than 65% of the samples had multiple infections by several Campylobacter species, including C. infans which has a high prevalence. This shotgun metagenomics technique successfully detects species that are difficult to culture and provides a more comprehensive picture of microbial communities in fecal samples. Although Campylobacter infection involves many species, the overall diversity of the gut microbiome does not show significant changes.

CONCLUSION

Based on the review of the literature, metagenomics demonstrates substantial promise as a diagnostic tool for diarrheal diseases, offering significant advantages over conventional culture-based and targeted molecular methods. Both 16S rRNA amplicon sequencing and whole-genome shotgun sequencing provide complementary insights—the former effectively characterizes community-level dysbiosis and ecological shifts in the gut microbiota, while the latter enables high-resolution detection of pathogenic species, co-infections, and antimicrobial resistance genes. Studies from India and Peru illustrate that metagenomics can uncover complex microbial interactions, identify novel or unculturable pathogens, and reveal resistome profiles that are critical for understanding disease etiology and transmission, particularly in endemic, resource-limited settings. The integration of these approaches into clinical and public

health frameworks could greatly enhance diagnostic accuracy, guide targeted therapies, and improve surveillance of diarrheal outbreaks.

For future research, it is recommended to conduct large-scale, longitudinal studies across diverse geographical and demographic populations to validate the clinical utility and cost-effectiveness of metagenomics in routine diagnostics. Efforts should focus on standardizing bioinformatics pipelines, reducing turnaround time, and making sequencing technologies more accessible in low-resource settings. Additionally, interdisciplinary studies combining metagenomics with host immune profiling, metabolomics, and epidemiological data could provide a more holistic understanding of diarrhea pathogenesis and inform the development of novel interventions, such as precision probiotics or targeted antimicrobial stewardship programs. Ultimately, fostering collaboration between clinicians, microbiologists, and bioinformaticians will be essential to translate metagenomic insights into actionable public health strategies that reduce the global burden of diarrheal disease.

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