REVIEW ARTICLE

Methodological Insights Into Gut Microbiome Profiling: A Comprehensive Review with Emphasis on Bipolar Disorder and Major Depressive Disorder

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ABSTRACT

Recently, evidence has linked alterations in gut microbiota composition with mental illnesses, particularly bipolar disorder (BD) and major depressive disorder (MDD). Previous human-gut microbiome studies have shown inconsistent results, and these inconsistencies may be attributed to the varying effects of methodological approaches on sample quality and data interpretation. This highlights the need for a comprehensive, standardised approach in gut microbiome research, especially regarding BD and MDD. In this methodological review, we evaluate the technical aspects of gut microbiota studies in BD and MDD conducted between 2018 and 2023, including sampling, DNA extraction, gene preparation, sequencing and bioinformatics analyses. Through evaluating existing methodologies, this review provides researchers with technical recommendations and standardisation guidelines to enhance the quality and consistency of future psychiatric microbiome research.
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INTRODUCTION

Bipolar disorder (BD) and major depressive disorder (MDD) constitute a substantial global health burden, affecting approximately 280 million and 40 million individuals, respectively. According to the World Health Organisation (WHO), BD is characterised by extreme mood fluctuations between depression and mania. At the same time, MDD manifests through persistent feelings of sadness, diminished interest in daily activities, and hopelessness (1). Both BD and MDD represent severe mood disorders necessitating precise diagnosis and individualised treatment (2). However, their overlapping symptoms frequently impede accurate clinical differentiation (3). Currently, no definitive diagnostic test exists for the early and precise identification of mood disorders. Clinical practice relies predominantly on comprehensive psychiatric assessments, mental state examinations, and routine laboratory and radiological investigations to exclude organic causes of mood disorders. Nevertheless, these methods occasionally lead to misdiagnosis and suboptimal treatment (4). Moreover, the aetiology of mental illness remains intricate and unclear, although researchers have identified several potential causes,

including limbic system damage, childhood trauma, and genetic factors (5-8). While scholars concur that these disorders emerge from multifaceted biological, psychological, and social factors, individual variations complicate the identification of a singular primary cause (9). Consequently, this complexity necessitates further research to elucidate the specific mechanisms influencing mental illness onset and progression.

One emerging research focus examines the correlation between gut microbiome and mental illness (5, 9, 10). Notably, researchers have observed substantial modifications in gut microbiota compositions among individuals with BD and MDD, suggesting the gut microbiome's viability as a biomarker for these disorders (11). This phenomenon stems from the gut microbiota's influence on bidirectional communication between the gut and brain through multiple pathways, encompassing neurochemical, neuroendocrine, immune systems, and metabolic mechanisms (11, 12). For example, gut microbiotas synthesise neurotransmitters, including acetylcholine and dopamine (9). Furthermore, studies have correlated gut dysbiosis with depression, potentially through processes such as gut wall permeability, bacterial translocation, and immunomodulation (13).

In examining the relationship between gut microbiota and BD and MDD, scientists commonly utilise sequencing techniques such as amplicon sequencing and whole genome sequencing (WGS) (10). These sophisticated methods have unveiled diverse gut microbial compositions in BD and MDD across studies. As illustrated in Table I, Zhang et al. (14) documented significant abundances of Megamonas, SMB53, and Enterococcus, whereas Huang et al. (15) identified elevated levels of Bacilli, Lactobacillales, and Veillonella in BD patients compared to healthy controls. However, these variations create challenges in identifying specific gut microbiota contributing to BD and MDD aetiology (5-8). While environmental factors, including geographical location, lifestyle, and dietary habits, influence these variations, methodological standardisation in human microbiome studies remains crucial for minimising discrepancies (16, 17).

Although several reviews address gut microbiota studies in BD and MDD, they lack a thorough methodological assessment, including the merits and limitations of various approaches throughout the research process (5, 17, 29). This review analyses methodological approaches employed to investigate gut microbiota diversity in individuals with BD and MDD from 2018 to 2023. We examine the progression of gut microbiota studies from faecal sample collection, processing, and storage through DNA extraction, 16S rRNA gene sequencing, and bioinformatic analysis. Additionally, we emphasise critical methodological considerations for gut microbiota research, specifically within the context of BD and MDD.

Table I: Gut microbiota compositions in BD and MDD patients

No	Authors	Location	BD and MDD associated Bacteria
1	(14)	China	BD: *Megamonas, SMB53, Enterococcus, Veillonella*
2	(15)	China	BD: *Bacilli, Lactobacillales, Veilonella* HC: *Dorea*
3	(18)	China	MDD severe I: *Bacteroides* D: *Ruminococcus, Eubacterium* MDD moderate I: *Bacteroides* D: *Fecalibacterium, Escherichia* HC: *Fecalibacterium*
4	(19)	China	BD I: *Lachnospiraceae, Oscillospiraceae, Bacteroideceae. Enterobacteriaceae* D: *Lachnospiraceae, Ruminococcaceae, Veillonellaceae, Prevotellaceae*
5	(20)	United States	No significant differences
6	(21)	United States	MDD *Eubacteriumsiraeum, Alistipes obesi, Holdemania filiformis, Lachnospiraceae bacterium 1.1.57FAA, Streptococcus salivariu*
7	(22)	Canada	No significant differences
8	(3)	China	MDD I: Bacteroidaceae, *Lachnospiraceae* D: *Bacteroidaceae* BD: I: *Lachnospiraceae, Streptococcaceae, Ruminococcaceae* D: *Lachnospiraceae, Prevotellaceae, Ruminococcaceae, Bacteroidaceae*
9	(23)	South Korea	MDD: *Prevotella 2, Ruminococcaceae UCG-002*
10	(24)	United States	MDD D: *Coprococcus catus, Clostridium symbiosum*

CONTINUE

Table I: Gut microbiota compositions in BD and MDD patients (CONT.)

No	Authors	Location	BD and MDD associated Bacteria
11	(25)	China	BD: *Parabacteroides, Bacteroides, Weissella, Halomonas* HC: *Roseburia, Fecalibacterium, Ruminococcus, Gemmiger, Parasutterella, Coprococcus*
12	(26)	Austria	BD: *Coriobacteriaceae, Enterobacteriaceae* HC: *Fecalibacterium*
13	(27)	Denmark	BD *Flavonifractor*
14	(28)	China	MDD Male: *Bacteroidetes* Female: *Actinobacteria*

Keys: -
I: increase, D: decrease, BD: bipolar disorder, MDD: major depressive disorder, HC: healthy control

GUT MICROBIOME SAMPLING, PROCESSING AND STORAGE TECHNIQUES

Given that the gastrointestinal (GI) tract harbours the largest population of microbes in the human body, various sampling methods have been developed, including biopsy, luminal brush, and others. Ideally, the approach should be non-invasive, minimise cross-contamination risk, avoid bowel preparation, and enable targeted sampling of multiple gut sites (30). Nevertheless, a faecal sample is widely utilised for gut microbiota studies due to its ease of collection and suitability for large-scale and longitudinal research (31, 32). Moreover, researchers in psychiatric fields often prefer fresh faecal specimens because they are naturally collected, non-invasive, and allow for repeated sampling, especially following numerous animal model studies demonstrating gut microbiota's influence on central nervous system development and function (5, 30, 33).

Despite the advantages of faecal sampling, several drawbacks exist, as reported by Tang et al. (30). First, faecal microbiota is unevenly distributed within samples and has its distinct biostructure. This heterogeneity leads to diverse microbiomes across different regions of such samples (34, 35). Wu et al. (36) agreed and emphasised the need to record faecal texture using the Bristol Classification chart. Their study found that firmer and denser faecal samples exhibit greater microbial composition, impacting microbial richness. Nevertheless, Carrillo et al. (35) indicated that vigorous homogenisation could effectively disrupt bacterial cells sufficiently, avoiding any bias in taxonomy. Second, while faecal material is generally considered representative of GI lumen contents, its composition may not accurately reflect direct interactions with the mucosa; hence, faecal samples cannot fully represent the composition and metagenomic function of mucosa-associated microbiota across multiple intestinal sites, potentially leading to biases when estimating intestinal microbiota with faeces (30). Additionally, Budding et al. (31) noted that faecal sample collection by individuals in their own environments can introduce potential contamination during the collection process.

Regarding specimen handling, while transporting raw faecal samples without preservatives, it is advisable to maintain a chilled temperature of 4°C to minimise alterations or degradation of the microbial composition (30, 36, 37). With proper handling methods, no significant change in microbial composition is expected within 24 to 48 hours of transportation to the laboratory (36, 37). However, the sample may also be kept at room temperature for up to four hours if the collection site is in proximity to the laboratory (38). Upon arrival, it is best to immediately aliquot samples or proceed with DNA extraction to avoid the stress of multiple freeze-thaw cycles that may cause significant changes to the bacteria's environment (36). Prompt freezing of faecal samples at -80°C is recommended to preserve microbial integrity for up to six months and is considered the gold standard for gut microbiota profiling (39). However, previous studies have shown that frozen faecal samples exhibited reduced levels of Bacteroidetes (40).

Thus, when immediate analysis or preservation at such temperatures is not feasible, chemical preservation methods can minimise the risk of microbiota degradation (32, 38). Several alternatives exist for storing faecal samples at room temperature for several days, including 95% ethanol, OMNIgene GUT Kit, RNAlater Stabilisation Solution, faecal occult blood test (FOBT) cards, and faecal immunochemical test tubes (30, 36, 41). Panek et al. (37) reported that faecal samples collected using the OMNIgene Gut Kit showed higher DNA quality and successfully preserved the Proteobacteria phylum, Prevotellaceae family, and Sutterella genus, comparable to fresh processed faecal samples. Wang et al. (41) further supported this finding, indicating that other preservation methods like FTA cards and RNAlater® were also effective in maintaining sample quality. Furthermore, the International Human Microbiota Standards (IHMS) also recommend the use of RNAlater Stabilisation Solution and storage at -20°C for faecal sample handling and storage methods (42).

Table II summarises microbiota studies among BD and MDD patients compared to healthy controls. Most studies collected faecal samples, except Rhee et al. (23), who used blood serum to study the human microbiome. For collection, Stevens et al. (21) and Coello et al. (27) used the OMNIgene Gut Faecal Collection Kit, while Madan et al. (24) utilised faecal swabs for sample collection.

Only three studies clearly mentioned their handling methods, keeping samples at 4°C with either an ice pack or dry ice (18, 20, 22). All studies preferred storing their samples at -80°C or -20°C for preservation, except for Painold et al. (26), which did not disclose their storage method. All the studies implemented practical sampling and storage methods to preserve faecal quality. Using similar faecal handling methods throughout a project for standardisation is advisable (36). This section highlights the critical importance of using fresh faecal samples and effective preservation techniques to maintain gut microbiome integrity in psychiatric research, a finding supported by animal studies.

Table II: Methodological summary of selected gut microbiota profiling studies in BD and MDD, 2018-2023

No	Author	No of patients	Sample type	Sample handling/ storage	DNA extraction kit	DNA quality	Gene sequencing method and platform	Hypervariable region and primer/ kit	Pipeline
1	(14)	58 BD 31 HC	Fresh faecal sample	Stored at -80°C	Magnetic Soil and Stool DNA Kit (Tiangen Biotech)	NA	16S rRNA IonS5TMXL	v3-v4 341F-806R	R software v4.1.0
2	(15)	72 depressed BD 16 HC	Fresh faecal sample	Stored at -80°C within 30 minutes	PSP® Spin Stool DNA Plus kit	1. AGE 2. Qubit® Fluorometer	16S rRNA Illumina MiSeq	v3-v4 341F-785R	USEARCH
3	(18)	138 MDD 155 HC	Fresh faecal sample	Freshly collected were transported at 4°C and within 6 hours to be stored in -80°C	E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA)	1. Nanodrop 2. 1% AGE 3. TBS-380 fluorometer	WGS Illumina NovaSeq	NA	NA
4	(19)	63 SCZ 50 BD 40 HC	Fresh faecal sample	Stored at -80°C within 30 minutes	HiPure Stool DNA Kits (Magen, Guangzhou, China)	1. Nanodrop 2. 1% AGE	16S rRNA Illumina NovaSeq PE250	v3-v4 338F-806R	USEARCH 8.0 UPARSE 7.1 QIIME v1.9.1 R software
5	(20)	110 MDD 27 HC 23 psy disorder other than MDD	Fresh faecal sample	Home: Transported with dry ice prior and to be stored in -80°C within 24hr Research visit: Stored at -80°C within 15-30 min	MoBioPowerSoil kit (Qiagen, Hilden, Germany)	NA	16S rRNA Illumina Miseq	v4 NEXTFlex® V4 Amplicon-Seq Kit 2.0	LotuS R software
6	(21)	18 hypertension 7 MDD 8 hypertension with MDD 21 HC	OMNIgene GUT faecal collection kit	Stored at -80°C immediately upon receiving from patient	NA	NA	WGS NA	NA	R software
7	(22)	17 BD	Fresh faecal sample	Transported with dry ice in insulated bag and to be stored in -80°C	QIAamp DNA Mini Kit (Qiagen, Valencia) Added with protocol from Sibley et al., 2011	1. Nanodrop	16S rRNA Illumina MiSeq	v3 341F-518R	In-house bioinformatic pipeline
8	(3)	165 MDD 217 BD 217 HC	Fresh faecal sample	Frozen	OMEGA-soil DNA Kit (Omega Bio-Tek, USA)	1. Nanodrop 2. 1% AGE	16S rRNA Illumina MiSeq	v3-v4 338F-806R	UPARSE 7.1
9	(23)	30 MDD 42 BD 36 HC	Serum	Stored at -80°C	DNeasyPowerSoil Kit (Qiagen, Germany)	QIAxpert system (Qiagen)	16S rDNA Illumina MiSeq	V3-v4 16S_V3_F-16S_V4_R	VSEARCH
10	(24)	81 MDD 16 BD 14 other psy illness	Fecal swab	Stored at -80°C	MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories)	NA	1. 16S rDNA 2. WGS Illumina MiSeq	v4 515F-806R	USEARCH v7.0.1090, UPARSE Casava v1.8.3
11	(25)	52 BD 45 HC	Fresh faecal sample	Stored at -80°C within 30 minutes	PSP Spin Stool DNA Plus Kit (Stratec, Berlin, Germany)	1. AGE 2. Qubit 2.0 Fluorometer	16S rRNA Illumina MiSeq	v4 341F-785R	Flash v1.2.11

CONTINUE

Table II: Methodological summary of selected gut microbiota profiling studies in BD and MDD, 2018-2023 (CONT.)

No	Author	No of patients	Sample type	Sample handling/ storage	DNA extraction kit	DNA quality	Gene sequencing method and platform	Hypervariable region and primer/ kit	Pipeline
12	(26)	32 BD 10 HC	Fresh faecal sample	Stored at -20˚C immediately	PowerLyzerPowerSoil DNA Isolation Kit (MO BIO Laboratories, CA, USA)	Picogreen fluorescence	16S rRNA Ion Torrent PGM	v1-v2 GATTGCCAGCAGCCGCGGTAA and GGACTACCAGGGTATCTAAT	USEARCH QIIME 1.8
13	(27)	113 BD 38 unaffected 1st degree relatives 77 HC	OMNIgene GUT faecal collection kit	Pre-paid mailers within 14 days and stored at −80 °C	NucleoSpin® 96 Soil kit (Macherey-Nagel) Vacuum protocol Added bead beating in cell lysis	NA	16S rRNA Illumina Miseq	v3-v4 341F-785R	USEARCH 10.0, mothur 1.38
14	(28)	Male: 20 MDD 20 HC Female: 24 MDD 24 HC	Fresh faecal sample	Stored at −80°C	PowerSoil kit Frozen samples pulverised using mortar and pestle in liquid nitrogen Added MoBio lysis buffer, garnet bead	NA	16S rRNA 454 pyrosequencing system	v3-v5 NA	Mothur 1.31.2

Keys: -
BD: bipolar disorder, MDD: major depressive disorder, Psy: psychiatry, SCZ: schizophrenia, HC: healthy control, NA: not available, WGS: Whole genome sequencing, AGE: agarose gel electrophoresis

DNA EXTRACTION METHODS FOR GUT MICROBIOME RESEARCH

DNA and RNA are essential for carrying genetic information within cells (43). Hence, efficient nucleic acid extraction is crucial for obtaining accurate results and successful amplification in organism detection (44). DNA extraction purifies DNA from a sample by separating it from other cellular elements using physical and/or chemical techniques (45). To standardise faecal microbiome studies, IHMS screened 21 DNA extraction protocols and recommends the repeated bead beating column (RBBC) with the utilisation of the Qiagen QIAamp DNA stool kit (46). However, this kit is no longer commercially available (47). Faecal DNA extraction typically involves four steps: sample homogenisation, cell lysis, impurity removal, and DNA purification (36). The homogenisation step is carried out to reduce the possibility of intra-sample variations, which could arise if the samples are prepared in batches, mainly for faecal samples of inconsistent texture (36, 48). However, according to Karu et al. (34), such variations might primarily affect the metabolic profile of the samples rather than the microbial profile. Plauzolles et al. (49) suggested that variations in the microbial profile within faecal samples are primarily influenced by the type of DNA stabiliser used and the highly individual-specific nature of the human gut, potentially overshadowing the influence of sample handling methods. Nevertheless, it is good practice to homogenise the samples first as a precautionary step.

A study in 2012 summarised various homogenisation methods such as grinding, shearing, beating, and shock-based techniques for various biological samples, including faecal specimens (50). While high-end homogenisation equipment like the Geno Grinder is effective, it can be costly in some laboratory settings. Therefore, modern sampling kits such as the OMNIgene GUT kit offer a more economical approach, enabling efficient homogenisation by shaking a few times or vortexing with a DNA stabiliser buffer (49, 51).

Cell lysis is crucial for extracting high-quality DNA with minimal degradation and accurately representing the microbial community. This can be achieved by disrupting cell membrane proteins, releasing encapsulated DNA, and breaking down glycosidic bonds within the cell membrane (51). Additional cell wall disruption is necessary when dealing with Gram-positive bacteria and fungi with robust cell walls via physical or chemical methods. Notably, physical disruption can yield higher DNA quantities, but precautions must be taken to prevent DNA fragmentation due to heat. While chemical or enzymatic methods are gentler, they may have limited applicability and introduce bias, particularly concerning target organisms (52).

Another aspect to consider while extracting DNA is the presence of PCR inhibitors (53). Removing these inhibitors is important to ensure the accuracy of DNA (44). PCR inhibitors, such as polysaccharides and degraded plant products, are often found in faecal samples and, being non-digestible, can affect the accuracy of the amplified DNA (54). Furthermore, degraded plant products, with physico-chemical properties similar to nucleic acids, can be co-purified together with DNA (55). While some kits, such as the QIAamp® DNA stool mini kit, include steps to remove these inhibitors, challenges remain due to the need for fibre intake in daily meals (54, 55).

Shin (44) and Zhao et al. (56) have summarised four widely known techniques to efficiently remove impurities and purify only the DNA, which include caesium chloride/ethidium bromide (CsCl/EtBr) density gradient centrifugation, phenol/chloroform extraction, solid-phase extraction, and the use of magnetic beads. While CsCl/EtBr density gradient centrifugation and phenol/chloroform extraction methods yield comparable results, they use toxic solvents, making them unsuitable for clinical labs.

To ensure a safer and user-friendly approach, researchers can opt for spin columns with solid-phase extraction techniques or magnetic beads, offering a safer and faster alternative. Most commercially available kits incorporate these recommended techniques for DNA purification, providing optimal results and ease of use across various laboratory settings (44, 56). While based on similar principles, kits differ in cell lysis and DNA purification methods (Table III), which allows researchers to choose the most suitable kit based on their budget and specific experimental requirements (57). Besides, most studies opt for commercial DNA extraction kits when conducting gut microbiota research among BD, MDD, and SCZ patients (5).

Table III: Commonly used faecal DNA extraction kits and their mechanism for cell lysis and DNA binding in DNA purification

Commercial DNA extraction Kit	Mechanical cell lysis (bead- beating)	Enzymatic lysis	Chemical lysis	Heat treatment	DNA binding
Macherey Nagel™ NucleoSpin™®-Soil	Yes	No	Yes	No	Silica membrane columns
Macherey Nucleospin Tissue	No	Proteinase K	Yes	Yes	Silica membrane columns
MagnaPure LC DNA isolation kit III	No	Proteinase K	Yes	Yes	Magnetic beads
MagPure Stool DNA	Yes	Proteinase K, RNase	Yes	No	Magnetic Beads
MO BIO PowerSoil® DNA isolation	Yes	No	Yes	Yes	Silica membrane columns
MP Bio FastDNA™ SPIN for Feces	Yes	No	Yes	No	Silica membrane columns
PSP® Spin Stool DNA Basic	Yes	Proteinase K	Yes	Yes	RTA Spin filter
QIAamp PowerFecal Pro DNA kit	Yes	No	Yes	No	Silica membrane columns
QIAamp® DNA Stool Mini	No	Proteinase K	Yes	Yes	Spin column
QIAGEN DNeasy PowerSoil Pro	Yes	No	Yes	No	Silica membrane columns
TianLong stool DNA/RNA extraction	No	Proteinase K	Yes	Yes	Magnetic beads
ZR Quick-DNA™ Fecal/Soil Microbe Miniprep	Yes	No	Yes	No	Silica membrane columns

Extracted DNA will be quantified using a spectrophotometer and visualised using agarose gel electrophoresis for its integrity analysis before PCR amplification. A260/280 ratio between 1.7 and 2.0 generally indicates good-quality DNA (44, 58). Church et al. (59) stated that PCR product sizes in the 100-200 bp range require approximately 1-3 ng of DNA, while larger products spanning 1000-2000 bp may necessitate higher quantities in the 10-40 ng range. This quality control step is crucial for an optimal DNA template for PCR by preventing the depletion of reagents or hindered data analysis due to low sample volume.

Table II shows the various commercial DNA extraction kits used in the studies, with the MO Bio PowerSoil® DNA isolation kit being the most popular (20, 24, 28), followed by the PSP Spin Stool DNA Plus Kit (15, 25). The remaining studies employed a range of kits (as listed in Table II), mostly adhering to the manufacturer's protocols. Only three studies modified their lysis step to increase DNA yield using: (i) bead beating method and the usage of Qiagen TissueLyser (22), (ii) bead beating step and the use of Vortex-Genie 2 (27), (iii) pulverising frozen samples using a mortar and pestle in liquid nitrogen aided by MO Bio garnet-bead tubes (28). Most studies assess DNA integrity using agarose gel electrophoresis and Nanodrop, with other alternatives including fluorometer (15, 18, 25), picogreen fluorescence (26), and QIAxpert system (23). Hence, this section has underscored the importance of selecting appropriate protocols and a DNA extraction kit to maximise DNA yield and integrity, ensuring reliable detection of microbial biomarkers relevant to BD and MDD, with the MO Bio PowerSoil® kit as the top choice among the 14 studies. The preferences for this kit may stem from its proven efficiency and superiority in DNA yield and purity over other kits (60).

16S rRNA GENE PREPARATION IN GUT MICROBIOME RESEARCH

DNA library preparation

Following DNA extraction, the next critical step in gut microbiota research is the preparation and sequencing

of the 16S rRNA gene. An effective library preparation for sequencing is crucial to maximise DNA fragment recovery, ensuring comprehensive genome coverage whilst conserving the amount of DNA that needs to be sequenced (61). The genomic DNA library preparation typically involves four steps: sizing or fragmentation of the target sequences to the desired length, ligation of oligonucleotide adapters to the fragment ends, amplification of the library, and measurement of the final product's concentration (62, 63).

Proper DNA sizing or fragmentation is essential for accurate sequencing, as shorter DNA fragments amplify more efficiently than longer ones (62). Enzymatic and physical methods are commonly used for DNA fragmentation. Whilst physical methods are potentially susceptible to cross-contamination and more expensive, enzymatic methods are versatile for various sample sizes but may raise concerns about batch-to-batch reproducibility. Nevertheless, both methods are considered acceptable and do not introduce bias in the results (63).

16S primer selection

The selection of PCR primers for DNA amplification constitutes the first step in gene sequencing studies (64). An ideal primer pair should maximise the entire microbial community while minimising PCR bias (51). Sambo et al. (65) stated that the primer pair comprises a forward primer, which aligns with the sense sequence of bacterial rRNA, and a reverse primer, which corresponds to the antisense sequence. Various genetic markers have been identified to help distinguish and categorise different microorganisms, and one of the most widely used is the 16S rRNA gene (66). Its small size (~1,542 bp) simplifies the sequencing process, even with large sample sizes (67). Additionally, this gene exists in all prokaryotic cells and contains both conserved and hypervariable regions, making it versatile for designing PCR primers, especially for next-generation sequencing (NGS) platforms (66, 68).

The 16S rRNA gene contains conserved regions, which are ideal for primer binding and capturing a broad range of bacterial taxa, and hypervariable regions that enable distinguishing between taxa in different microbial environments and identifying organisms at the genus or species level. These features enable the determination of bacterial communities' relative compositions in a sample, even when dealing with microbes that are difficult to culture (69-71).

One of the widely adopted primer sets for exploring bacterial diversity is the 341F/785R, targeting V3-V4 hypervariable regions of the 16S rRNA gene (72, 73). However, a 2019 study found that the 515F/806R primer set, focusing on the V4 region, increases alpha diversity, specifically in gut communities (74). Whilst most studies on MDD target the V3 and/or V4 regions of the 16S rRNA gene, the diversity in primer selection often leads to inconsistencies in reported microbial profiles (17).

Whilst nine hypervariable regions exist in the 16S gene, the V4 and V2 regions demonstrate the lowest error rates in assigning taxonomy compared to other regions (75). The V4 region also provides comparable and unbiased results across various sequencing methods and enables the use of 300-bp sequences. Furthermore, combining the V4 and V3 regions in paired-end sequencing generates longer, higher-quality reads, thereby enhancing the accuracy of taxonomy classification (74).

PCR product purification and quantification

While preparing the target gene, PCR products were potentially contaminated with various PCR contaminants and PCR artefacts, ultimately leading to PCR biases (76). These contaminants and artefacts may include DNA extraction reagents, eukaryotic DNA, primer mismatches, primer dimers, and several others (51, 76, 77). Consequently, a purification step for PCR products is essential, especially given the sensitivity of sequencing methods (51).

DNA purification can be achieved through multiple methods, including magnetic bead purification, enzymatic purification, and gel purification (51, 59). Various commercially available library preparation kits can simplify this process by capturing DNA fragments larger than 100 bp and those smaller than 50 bp. However, these parameters can be adjusted to suit the specific library size of interest (51, 59). Moreover, some commercial kits, like the BigDye kit, allow skipping the post-PCR purification step, as they come with primers suitable for both 16S amplification and sequencing (59). In comprehensive reviews by Kapp et al. (78) and Silva et al. (51), it is essential to quantify and combine samples equally after the purification steps to ensure each library component is evenly represented in the ultimate pool. Quantification using qPCR is preferred for purified amplicons compared to spectrophotometers, as it is more precise since it explicitly measures the amplicon-based on the intercalating dyes. During the final library preparation phase, the amplicons must be pooled at similar molar concentrations, ideally at a minimum of 4 nM, to avoid low data yield.

In Table II, 13 studies employed 16S rRNA sequencing to explore gut microbiota diversity, targeting the V1 to V5 region from the 16S rRNA gene. Notably, the V3-V4 region was frequently examined, with seven studies utilising this region (3, 14, 15, 19, 23, 27). The V4 region ranked second-most sequenced and was featured in two studies (20, 24, 25). As for primers, three studies used 341F/785R (15, 24, 25, 27), two used 338F/806R primer (3, 14, 19), and the others varied. Although the V3 and V4 regions are commonly targeted in BD and MDD studies, variations in primer selection can lead to inconsistent microbial profiles. Standardising primer

choice, such as using the 341F/785R set, is essential for comparability across studies.

16S rRNA SEQUENCING IN GUT MICROBIOME RESEARCH

Evolution of gut microbiome studies

Early exploration of the human gut microbiome relied upon cell culture techniques. However, these methods were constrained, offering limited understanding due to their inability to comprehensively explore microbial taxa and elucidate complex microbial interactions, primarily because only a small fraction of microorganisms are culturable (79, 80). The introduction of several microbiome analyses, such as amplicon sequencing, shotgun metagenomics, metabolomics, metaproteomics, and metatranscriptomics, has revolutionised the field, allowing a significantly more profound understanding of the microbiome (81, 82).

Amplicon sequencing and shotgun metagenomics are typically the most commonly used methods for sequencing the genomic material of the microbiome (82). Amplicon sequencing targets specific gene markers, whilst shotgun metagenomics sequences all microbial genomes available in a sample (83). Both methods provide information for identifying taxa and determining the functional potential of microbial communities, making them suitable for gut microbiome analysis, as evidenced in a review by Knudsen et al. (17).

In amplicon sequencing, commonly used markers include the 16S rRNA gene for bacteria, 18S rRNA, and internal transcribed space (ITS) for fungi and other eukaryotes (81, 82). The 16S rRNA gene amplicon sequencing remains the most popular method due to its cost-effectiveness, rapid analysis, and applicability across various research scales (82). However, limitations associated with this method include challenges in selecting appropriate primers, sensitivity to primer and region choices leading to potential taxonomic bias, and taxonomy assignment limited to genus level (81-83).

Shotgun metagenomics is preferred when more comprehensive information is needed within a sample, as it can sequence the whole genome, including fungi and bacteria, though dependent on reference genomes (81). Additionally, this technique is preferred when species-level taxonomic resolution is required (82, 83). However, this method has several drawbacks, including high operational costs, requirements for high-quality sequence reads, intensive computational analysis, and difficulty implementing in general laboratories (80, 84). Nevertheless, due to 16S gene sequencing limitations causing inconsistencies in capturing the full range of gut microbiota, research in BD and SCZ is shifting towards shotgun metagenomics, which offers more comprehensive microbial profiling (85).

Metatranscriptomics, metaproteomics, and metabolomics are preferred for a deeper understanding of microbial activity expression (81). Metatranscriptomics, another advanced method, discovers the whole microbiome community and determines its functional profile under specified conditions, which are often influenced by host status (86). Proteomics generally refers to protein studies, whilst metaproteomics studies proteins from multiple organisms (87). Metabolomics studies aim to characterise microbiota-generated metabolites and examine their interactions with both microbiota and host metabolism (81).

Gene sequencing platform

To perform either amplicon sequencing or shotgun metagenomic sequencing, a sequencing platform such as Illumina MiSeq or Ion Torrent PGM56 is needed (59). Diverse sequencing platforms are available in the market due to progressive improvements made to decode the genetic data of microbiomes from both RNA and DNA perspectives (79, 88). These platforms have evolved through three generations of sequencing technology: Sanger sequencing as the pioneer, NGS as the second generation, and third-generation sequencing (TGS) as the latest generation (61, 80, 89). Notably, NGS has significantly contributed to advancements in genomic research and clinical diagnostics, including the study of psychiatric and psychological disorders, as it enables beneficial multi-omics approaches (90).

The pioneer generation involves the selective integration of chain-terminating ddNTPs by DNA polymerase, enabling the identification of specific bases within DNA strands (89). While newer platforms have emerged, Sanger remains relevant due to its ability to produce longer read lengths and is considered the gold standard for investigations focusing on small regions of genomic targets (88, 89). NGS, a high-throughput sequencing (HTS) method, enables rapid and cost-effective processing, with examples like Roche 454 sequencing, Illumina sequencing, and Ion Torrent PGM sequencing (80). In the NGS era, the principles were divided into sequence by synthesis and sequence by hybridisation (91). Third-generation sequencing employs single-molecule sequencing that allows for longer read lengths in real-time with several mechanisms, including sequence reads through excitation of fluorescent dNTPs and ion current changes (61, 79, 88, 89). Examples include RSII and Sequel from PacBio and nanopore sequencing. Additionally, both PacBio sequencing and nanopore sequencing do not require PCR amplification steps (61).

Illumina platform

When sequencing the 16S gene, the Illumina platform was preferred over other platforms, and this platform, particularly MiSeq and HiSeq, has been responsible for sequencing up to 82% of bacterial genome RefSeq

(92, 93). Additionally, the HMP employed the Illumina platform to identify microbial communities, including gut microbiota (79). A review by Vindegaard et al. (29) highlighted Illumina MiSeq as the leading choice for gut microbiota studies in the psychiatric field, including BD and MDD. The preference was due to its cost-effectiveness and capacity to deliver ultra-high throughput and speed, surpassing alternatives like Sanger and Roche 454 sequencing. However, the Illumina platform has limitations, notably its relatively short read length of 100-600 bp. This short read length can introduce potential species-level bias and lead to difficulties in identifying novel species (88).

Illumina has consistently enhanced its instruments for competitiveness, improving read quantity and quality by reducing error rates to generate valuable high-throughput 16S amplicon data. The HiSeq system addressed sequencing errors and substitution biases found in the Genome Analyzer (GA and GA IIx), significantly lowering error rates from 0.76% to 0.26%, thus achieving HTS (94, 95). In 2011, the MiSeq system offered smaller laboratories and the clinical diagnostic market more affordable per-sample costs and user-friendly operation, though with lower throughput (95, 96). The latest NovaSeq further reduced error rates, leading to the discontinuation of HiSeq (94, 97).

Table II indicates that one study applied advanced WGS (18), two conducted both WGS and amplicon sequencing (21, 25), while the other 12 studies used 16S rRNA/rDNA gene sequencing. Illumina was the prevailing platform, with eight studies using Illumina MiSeq (3, 15, 20, 22-25, 27) and two using Illumina NovaSeq (18, 19). IonS5TMXL (14) and Ion Torrent PGM (26) are two additional used models from Ion Torrent sequencing platforms. Only one study employed Roche 454 sequencing (28). The selection of this platform was less common, possibly due to Roche's decision to cease operations in 2013 (89). Besides these NGSs, two studies started utilising Illumina NovaSeq, a TGS, to sequence their DNA samples (18, 19). Nevertheless, the consensus remains that despite the emergence of TGS, Illumina and other NGS platforms remain relevant in this domain.

BIOINFORMATIC ANALYSIS IN GUT MICROBIOME RESEARCH

Bioinformatic analysis pipelines for 16S sequencing

Various software tools analyse sequence reads to create bioinformatic pipelines. QIIME 2 supports amplicon sequence variant (ASV) picking algorithms, such as DADA2 and Deblur in R. Other options include Mothur, DADA2, and USEARCH with UPARSE and UNOISE algorithms (51, 71). Although USEARCH is commonly used, it is not open-source software; alternatively, VSEARCH can be utilised (51). Bioinformatic pipelines of amplicon analysis steps involve raw-read quality control, demultiplexing, merging, eliminating low-quality reads, detecting and removing chimeric sequences, denoising sequences of ASV or operational taxonomic unit (OTU), and taxonomically classifying them (82). Quality control was assessed throughout the analysis and measured using the Phred quality score, where higher scores indicate less probability of incorrect base calls (51). Each pipeline utilises different quality control parameters, including primer removal, off-target outlier sequence read removal, removal of poor-quality reads based on Phred scores, and removal of reads exceeding a defined maximum number of expected errors (51, 82).

Abellen-Schneyder et al. (71) and Liu et al. (82) explained that during amplicon analysis, selecting representative sequences for species taxonomy assignment requires consideration. Two predominant approaches exist: clustering into OTUs and denoising into ASVs. While OTUs cannot capture subtle differences among species or strains even at 97% similarity to UPARSE, ASVs using the DADA2 denoising algorithm represent sequences with higher precision. Zero-radius OTU provides another alternative where this clustering method contains threads originating only from identical bacterial species, enabling cross-study comparison. After denoising or clustering, sequences receive taxonomic classification using databases of known 16S rRNA gene sequences, such as GreenGenes, Ribosomal Database Project (RDP), SILVA, or All-Species Living Tree (71). Database selection is crucial since only a few, like SILVA and RDP, receive regular updates; consequently, these two are widely used in various gut microbiota studies, including BD and MDD gut microbiota profiling (16, 17).

Microbial diversity analysis

The significance of determining bacterial diversity in gut studies lies in its correlation with individual health, where higher bacterial diversity is always associated with better health (79). Essentially, gut microbial diversity can be evaluated using α-diversity and β-diversity metrics (51).

The α-diversity refers to the diversity of bacteria and their uniformity within a sample, such as faecal or saliva. Chao1 and the number of observed OTUs/ASVs are the metrics commonly utilised to indicate species richness or diversity. Meanwhile, the distribution of different taxa to other species within a sample can be assessed through the Evenness index (51). Additionally, there are indices that combine both richness and evenness, such as the Shannon Index and inverse Simpson (32). The Shannon index can characterise the presence of rare species within a sample, indicated through an index value of more than five, and a higher value signifies that the sample has greater α-diversity abundance. As for the inverse Simpson, this index is more influenced by the common species. Another method for assessing diversity involves phylogenetic richness estimators,

such as Faith's phylogenetic diversity (81). Moreover, these indices can also be represented in rarefaction curves and further developed into a scatter plot against environmental measures. The results obtained from the scatter plot will provide an understanding of the factors influencing the diversity (51).

β-diversity refers to microbiota composition differences between two samples or groups (32). This analysis provides insight into how diversity among samples can alter the microbiota over time, across different health conditions in hosts, or in response to specific factors of interest (51). Bray-Curtis dissimilarity and UniFrac distance are commonly used β-diversity indices. Bray-Curtis dissimilarity is a quantitative measure of compositional dissimilarity between two samples or groups, ranging from zero (similarity) to one (dissimilarity) (32). Meanwhile, UniFrac distance measures differences between groups based on phylogenetic relationships. It comes in two forms: unweighted UniFrac, which considers the presence or absence of taxa and is sensitive to changes in the richness of rare species, and weighted UniFrac, which accounts for abundance information while giving less weight to rare species. The Jaccard index is another qualitative measure focusing on feature presence or absence, similar to unweighted UniFrac (32, 81).

To further validate the findings in β-diversity analysis, permutational multivariate analyses like PERMANOVA can be utilised. For visualisation, ordination methods such as non-metric multidimensional scaling, principal coordinate analysis, or canonical correspondence analysis are recommended (51). A permutation test p-value of less than 0.05 indicates a statistically significant β-diversity between different communities (98).

Analysing associated metabolic pathways is highly recommended to gain insights into the pathophysiology of diseases from a microbiome perspective (99). To predict the impacted functional or metabolic pathways, the utilisation of PICRUSt2 is recommended, and the information obtained is often compared with metabolic pathways listed in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. PICRUSt2 is another bioinformatics software package specifically designed to predict metagenome function in both marker genes and the complete genome of the microbiome (99, 100). The KEGG contains a diverse collection of data related to biological systems and is regularly updated (82).

Table IV shows that the most popular pipeline is the USEARCH pipeline, with five users (15, 19, 24, 26, 27), followed by the R pipeline with four users (14, 19-21). In assigning taxonomy for the sequences, there are five choices included: RDP (3, 19, 25, 27, 28), SILVA (19, 20, 22, 23), GreenGenes (14, 26), NCBI database (18), and the MetaPhlAn2 marker gene database (24). In investigating the α-diversity, the Shannon index was widely used in most studies, followed by Chao1 and Simpson. Notably, there is also Evenness with one user (18), two for incidence-based coverage estimator (ICE) (15, 25), and four for abundance-based coverage estimator (ACE) (3, 14, 19, 20). In β-diversity measurement, six studies used Bray-Curtis dissimilarities (3, 14, 18-20, 23) and UniFrac distance (19, 20, 23, 25-27), two studies used Aitchison distance (20, 21), and one user utilised Hellinger-Shannon divergence and Jaccard index (24). Five studies further explored the genomic functional pathway through PICRUSt (14, 19, 25), HUMAnN2 (24), or Tax4Fun (23) to further examine the microbial functional profile. Nevertheless, the KEGG database was eventually used as a reference database. In this bioinformatics analysis section, we revealed that the USEARCH pipeline and RDP database are the preferred choices for decoding microbial profiles in BD and MDD research, valued for their reliability and compatibility with high-throughput sequencing data in psychiatric research outcomes.

Table IV: Summary of bioinformatic analyses for selected gut microbiota profiling studies in BD and MDD, 2018-2023

No	Author	Taxonomic database	α-diversity	β-diversity	β distance analysis	Functional profile/ database
1	(14)	GreenGenes v13_8	1. Shannon 2. Simpson 3. Chao1 4. ACE	1. Bray-Curtis	1. PCoA 2. PERMANOVA	PICRUSt/ KEGG
2	(15)	ND	1. Shannon 2. Simpson 3. Chao1 4. Observed OTUs 5. Inverse Simpson 6. ICE	ND	1. PCoA	NA
3	(18)	NCBI database	1. Shannon 2. Simpson 3. Dominance 4. Evenness	1. Bray-Curtis	1. PCoA 2. PERMANOVA	ND/ KEGG

CONTINUE

Table IV: Summary of bioinformatic analyses for selected gut microbiota profiling studies in BD and MDD, 2018-2023 (CONT.)

No	Author	Taxonomic database	α-diversity	β-diversity	β distance analysis	Functional profile/ database
4	(19)	1. RDP classifier v2.2 2. Silva v138	1. Shannon 2. Simpson 3. Chao1 4. Observed species 5. ACE 6. Phylogenetic diversity	1. Bray-Curtis 2. UniFrac	1. PCoA 2. PERMANOVA	PICRUSt2/KEGG
5	(20)	Silva	1. Shannon 2. Chao1 3. Observed OTUs 4. ACE 5. Phylogenetic diversity	1. Bray-Curtis 2. UniFrac 3. Aitchison	1. PCoA 2. PCA 3. PERMANOaA	NA
6	(21)	ND	NA	1. Aitchison	1. PERMANOVA	NA
7	(22)	Silva v1.38	1. Shannon 2. Simpson	NA	NA	NA
8	(3)	RDP	1. Shannon 2. Chao1 3. ACE	1. Bray-Curtis	1. PERMANOVA	NA
9	(23)	Silva 128	1. Shannon 2. Chao1 3. Observed OTUs 4. Inverse Simpson	1. Bray-Curtis 2. UniFrac	1. PCoA 2. PERMANOVA	Tax4Fun/ND ND/KEGG
10	(24)	MetaPhlAn2 marker gene database	1. Shannon 2. Observed OTUs	1. Jaccard	1. PCA	HUMAnN2
11	(25)	RDP v2.12	1. Shannon 2. Simpson 3. Chao1 4. Observed OTUs 5. Inverse Simpson 6. ICE	1. UniFrac 2. Hellinger 3. Jensen-Shannon divergence	1. PCoA 2. PERMANOVA	PICRUSt/ KEGG
12	(26)	GreenGenes v13_8	1. Shannon 2. Simpson 3. Chao1 4. Observed species	1. UniFrac	1. PCoA	NA
13	(27)	RDP training set v16	1. Shannon 2. Observed OTUs	1. UniFrac	1. PERMANOVA	NA
14	(28)	RDP	1. Shannon 2. Simpson 3. Obs OTUs 4. Phylogenetic diversity	NA	1. PCoA	NA

Keys:-
ACE: abundance coverage estimator, OTU; operational taxonomy unit, ICE: incidence coverage estimator, KEGG: Kyoto Encyclopedia Genomes and Genes, NA: not available, ND: not defined

CONCLUSION

This review paper highlighted the challenges in gut microbiota research for BD and MDD, particularly emphasising the necessity for standardised methodologies to ensure reproducible data. Despite the limited studies reported in the past five years, our compilation reveals researchers' preference for the MO Bio PowerSoil® DNA isolation kit in DNA extraction from fresh faecal samples, amplicon sequencing targeting V3/V4 regions of 16S rRNA gene using primer 341F/785R, and implementation of USEARCH pipeline with RDP database for sequence analyses. The bioinformatic analyses incorporated the Shannon index for α-diversity, Bray-Curtis dissimilarity and UniFrac for β-diversity, and functional analysis via PICRUSt based on the KEGG database. This compilation offers preliminary guidance for standardised methodology in gut microbiota studies focusing on BD and MDD, as these approaches demonstrate coherence and widespread acceptance. Moving forward, developing a comprehensive protocol for gut microbiota studies in BD and MDD would enhance reproducibility and consistency across investigations in this field.

In addition to improving result reproducibility through methodological standardisation, future studies should consider incorporating multi-omics approaches and multiple technologies to elucidate the mechanisms underlying gut microbiota's role in mental illness. Through these integrative approaches, researchers can examine microbial protein and gene expression profiles while accounting for confounding variables such as daily routines, medications, and demographics, thereby advancing predictive diagnostic methods to differentiate between healthy individuals and patients.

In conclusion, addressing the heterogeneity in analytical methods highlights the necessity for standardised guidelines in sample collection, processing, and analysis. Establishing such guidelines would create substantial opportunities for advancing our understanding of BD and

MDD aetiologies. This advancement could yield novel insights, innovative screening tools, and alternative therapeutic approaches for individuals affected by these complex disorders.

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