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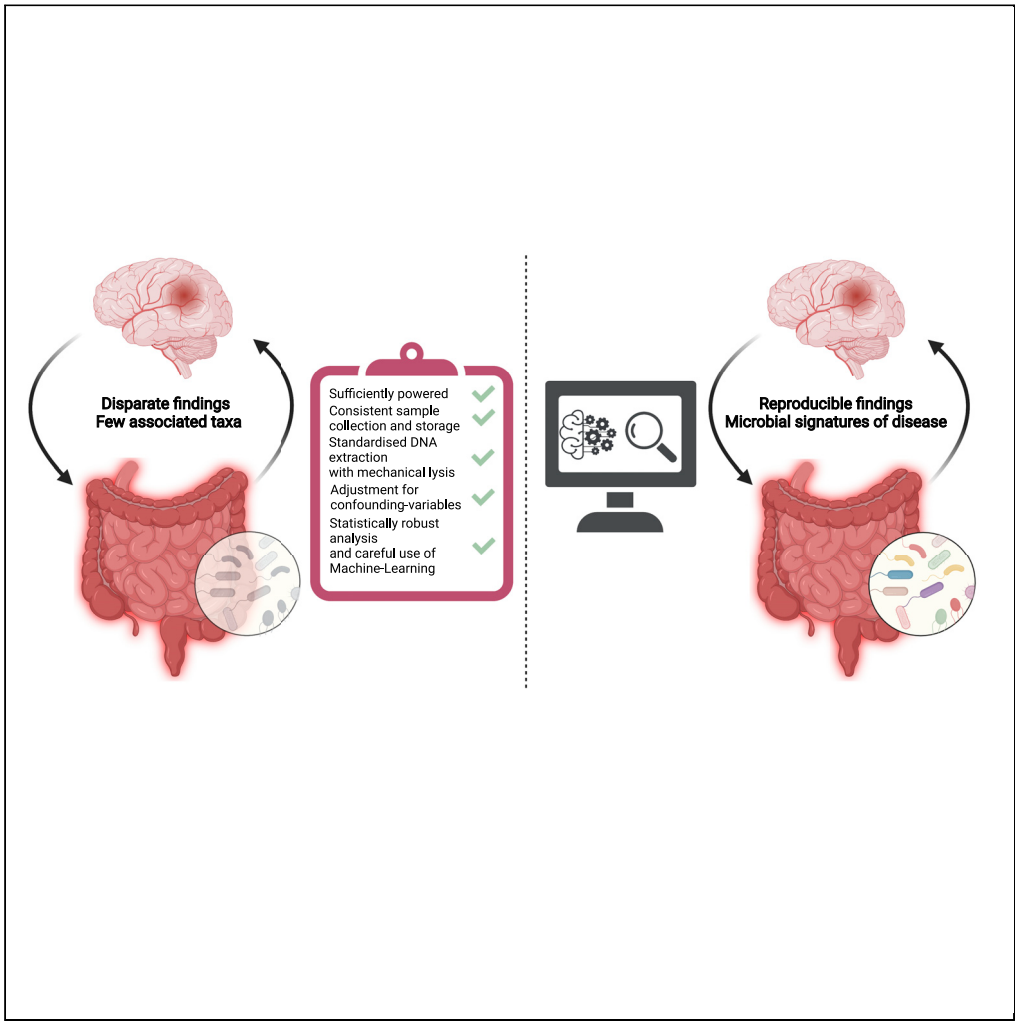
Increasing transparency and reproducibility in stroke-microbiota research: A toolbox for microbiota analysis



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Highlights

Guidelines for reproducible stroke-microbiota research in patients and animal models

Current best practices for 16S rRNA profiling and analysis

Easy-to-use, freely available bioinformatics pipeline for gut microbiota analysis

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Increasing transparency and reproducibility in stroke-microbiota research: A toolbox for microbiota analysis

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SUMMARY

Homeostasis of gut microbiota is crucial in maintaining human health. Alterations, or "dysbiosis," are increasingly implicated in human diseases, such as cancer, inflammatory bowel diseases, and, more recently, neurological disorders. In ischemic stroke patients, gut microbial profiles are markedly different compared to healthy controls, whereas manipulation of microbiota in animal models of stroke modulates outcome, further implicating microbiota in stroke pathobiology. Despite this, evidence for the involvement of specific microbes or microbial products and microbial signatures have yet to be identified, likely owing to differences in methodology, data analysis, and confounding variables between different studies. Here, we provide a set of guidelines to enable researchers to conduct high-quality, reproducible, and transparent microbiota studies, focusing on 16S rRNA sequencing in the emerging subfield of the stroke-microbiota. In doing so, we aim to facilitate novel and reproducible associations between the microbiota and brain diseases, including stroke, and translation into clinical practice.

INTRODUCTION

Besides select sterile sites, the human body is colonized by complex communities of microbes, termed the microbiota that encompasses bacteria, fungi, and viruses. These microbial communities have co-evolved with us playing a crucial role in health and disease (Claesson et al., 2017). Characterization of the microbiota has been the subject of intensive research in the past decade, generating large volumes of data and with the advent of next-generation sequencing and bioinformatic methods to analyze such data sets; these communities are being characterized in-ever greater detail. Microbiota data, however, are highly complex and high-dimensional, and affected by a multitude of confounding factors; thus, careful consideration of sample collection, study design, and bioinformatic analysis is necessary to ensure robust and reproducible results (Ghosh et al., 2020; Vujkovic-Cvijin et al., 2020).

Analysis of high-throughput microbial data often requires specialist training but progress has been made on lowering the barrier of entry, particularly for biologists who may lack the statistical or bioinformatic expertise necessary to conduct these analyses (Bolyen et al., 2019; Lagkouvardos et al., 2016; Schloss et al., 2009). Although commendable, this, in combination with the surge in attention surrounding the microbiota, has led to an increase in underpowered, associative, and often overinterpreted studies that limit translational impact (Schloss, 2018). Fecal microbiota transplantation (FMT) and probiotics are the only microbiota-based therapies currently used in clinical practice. FMT is solely used to treat *Clostridioides difficile* infection, whereas despite widespread use of probiotics, evidence regarding their efficacy as a treatment is currently lacking (Gupta et al., 2016). Clinically recommended uses are currently limited to formulations containing *Lactobacillus* and *Bifidobacterium* to prevent necrotizing enterocolitis in preterm infants (Su et al., 2020). Regardless, the recent adoption and development of orthogonal methods to characterize microbiota, such as gnotobiotic mice and metabolomics have allowed elucidation of the mechanisms underlying microbiota-disease associations (Chu et al., 2019). Fully embracing these methods and ensuring microbiota studies are well designed will ensure further development and establishment of emerging subfields of microbiota research, especially in fields addressing complex gut-brain interactions, such as the involvement of the microbiota in ischemic stroke (Delgado Jiménez and Benakis, 2021).

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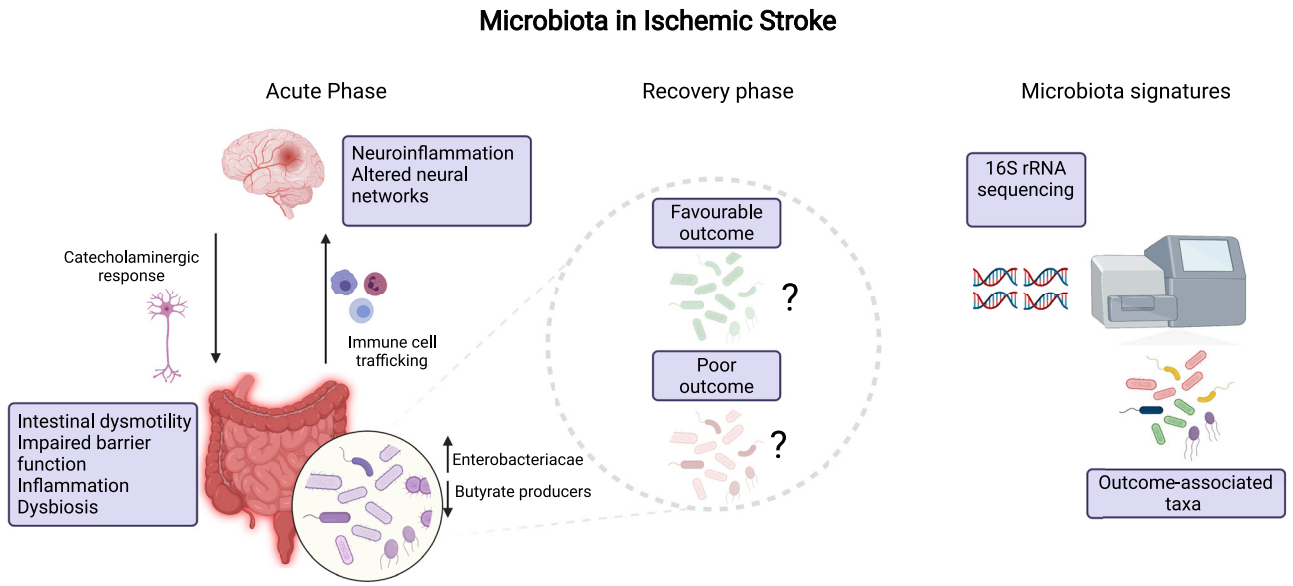

Figure 1. The gut microbiota in ischemic stroke

A summary of current knowledge on the involvement of the gut microbiota in stroke and outstanding questions which microbiota signatures identified by 16S rRNA sequencing can answer (created with BioRender.com)

Recently, the interaction between the gut microbiota and CNS – the gut brain axis – has been implicated in the etiology of neurological diseases, particularly those with a neuroinflammatory basis such as cerebral infarction (Benakis et al., 2016; Cryan et al., 2020; Houlden et al., 2016; Singh et al., 2016; Xu et al., 2021). Indeed, patients with severe stroke display significantly altered microbiota compared to healthy age-matched controls. Xia et al. identified dysbiotic communities in stroke patients, with the level of dysbiosis predicting stroke severity (Xia et al., 2019). In murine studies, manipulation of microbiota with antibiotics before experimental stroke improved the outcome. Further, the microbiota affects the development of brain lesions by regulating polarization of gut T cells that traffic to the brain post-stroke (Benakis et al., 2016, 2020). Despite the implication of the microbiota in stroke severity and outcome, identification of both taxonomic signatures and functional mechanisms of disease remains to be seen. Uncovering a stroke-associated microbiota signature holds the potential to utilize specific gut bacteria as therapeutic interventions, impacting host pathobiology and stroke outcome (Figure 1). High-level associations such as an increase in members of Enterobacteriaceae and a decrease in butyrate-producing bacteria have been shown by several groups but insights at lower taxonomic resolution are lacking (Haak et al., 2021; Xu et al., 2021). Two recent studies, however, have linked specific microbes or their metabolites to stroke severity and outcome. Post-stroke expansion of *Escherichia coli* and immune stimulation via lipopolysaccharide was linked to systemic inflammation and impaired outcome (Xu et al., 2021). High levels of the metabolite trimethylamine-N-oxide (TMAO) – a gut microbial metabolite known to be associated with cardiovascular disease – increased infarct volume and led to a poorer functional outcome (Zhu et al., 2021). Together, these studies provide significant evidence for an important role of the microbiota in stroke pathogenesis and treatment, yet a distinct microbial signature has not been identified, hampered by disparate findings between existing studies, likely owing to differences in methodologies, low sample sizes, and unacknowledged confounding variables (Sadler et al., 2017; Singh et al., 2016; Stanley et al., 2018).

In general, existing stroke studies have been conducted to a high methodological standard. Nevertheless, more carefully designed and conducted investigations into the role of the microbiota in stroke will enable reproducibility between different studies and further generation of mechanistic insights. This review aims to provide a framework for conducting well-designed microbiota studies in general, but with a focus on aspects specific to stroke research. Furthermore, we aim to facilitate cross-study comparison and reproducibility of stroke-microbiota studies, supporting translation into clinical practice. Importantly, this work presents a publicly available toolbox and reproducible bioinformatic pipeline to analyze 16S rRNA gene sequencing data applicable to all microbiota studies.

Box 1. Recommendations for sample collection and processing

Design	Recommendations	Further reading
Sample size	Consult a statistician or utilize tools such as powmic or Micropower to estimate sample size before beginning study	(Chen, 2020; Debelius et al., 2016; Kelly et al., 2015)
Sample collection method	Stool samples: fresh sample Tissue samples: whole biopsies rather than mucosal scrapes are preferable	(Claesson et al., 2017; Liang et al., 2020)
Sample storage	Store samples immediately at −80°C or if study design requires RT storage, store in 95% ethanol	(Marotz et al., 2021; Pollock et al., 2018)
DNA extraction method	Use a mechanical lysis method and try to ensure samples are processed with the same kit	(Gerasimidis et al., 2016; Nearing et al., 2021)
Controls	Prudent use of negative and positive controls. We recommend at least one extraction control per batch and additional water controls during library preparation and sequencing	(Bedarf et al., 2021; de Goffaude et al., 2018)
Sequencer	Illumina MiSeq/HiSeq™ machines are appropriate for most 16S studies	(Caporaso et al., 2012; Pollock et al., 2018)
Hypervariable region	V1-V2/V3, V4, and V3-V4 are all commonly used and suitable for animal or human studies	(Abellan-Schneyder et al., 2021)

Pitfalls in stroke-microbiota studies

Each step in the process of conducting a microbiota study can introduce bias, impacting downstream results. The sheer variety in sample collection methods, DNA extraction, and sequencing itself can be overwhelming for those new to the field. By discussing each step in-detail, outlining where problems can occur and how, and suggesting best practices while also providing a detailed step-by-step microbiota analysis from stroke fecal samples, we aim to provide a set of guidelines and our own recommendations (Box 1) for stroke researchers who wish to enter the field, enabling them to conduct well-designed and executed studies. Importantly, we believe our guidelines and bioinformatic pipeline will be of interest beyond the field of microbiota stroke research.

Study design

Sample size and experimental design is one of the most important steps in conducting a microbiome study but often overlooked (Debelius et al., 2016). For human microbiota analysis, it is crucial to define the type of study (i.e., cross-sectional, or longitudinal) and the question of interest as this will impact processing and analysis downstream. A cross-sectional study of mucosal biopsies will require different extraction methods and controls than a longitudinal follow-up study of stool samples. Thus, the experimental design needs to consider the following points: (1) Will this be a longitudinal or cross-sectional study? (2) How will samples be preserved and within what time frame will they be processed? (3) Which readouts are planned, 16S, Shotgun metagenomics, Metabolomics or multiple? If using 16S which hypervariable region will be used? (4) Is the study well powered and controlled? (5) Are potential confounding variables well documented and can they be accounted for? By ensuring all these questions are sufficiently answered, robustness and reproducibility of results can be significantly improved.

Many confounding variables can impact microbiome composition, potentially obscuring genuine patterns or leading to false conclusions. Factors such as lifestyle (Vujkovic-Cvijin et al., 2020), geography (He et al., 2018), diet (Asnicar et al., 2021a), and medication (Forslund et al., 2021) all impact microbiota composition. In stroke patients, stool transit time and frequency, sex, age, and smoking may also confound results, either alone or in an additive or combinatorial manner. Importantly, many of these factors are either

Table 1. Experimental design considerations for human studies

Considerations	Recommendations
Co-morbidities	Record any significant existing or prior medical conditions
Age	Ensure control groups are age matched
Sex	Stroke severity and outcome is often worse in women and may need to be adjusted for (Phan et al., 2019)
Geography	Ensure individuals reside in the same region/country or stratify by location (He et al., 2018)
Food intake	Patients with severe stroke may have reduced food intake, potentially confounding results (FOOD Trial Collaboration, 2003)
Stool consistency	Record stool consistency if possible (Lim et al., 2012; Vujkovic-Cvijin et al., 2020)
Infection	Post-stroke infections are common and should be recorded (Chamorro et al., 2007)
Medication	Existing medication may confound results, patients with a history of antibiotic use in the past six months should be excluded (Forslund et al., 2021; Vujkovic-Cvijin et al., 2020)
Treatment	Treatment given – i.e., Thrombolysis should be recorded and tested for an effect on the microbiota. Surgery likely additionally impacts microbiota and should be noted
Alcohol use/smoking	History of alcohol use and smoking are independent stroke risk factors but may also impact microbiota composition (Hankey, 2020; Vujkovic-Cvijin et al., 2020)

impacted by or are risk factors for stroke themselves (Asnicar et al., 2021b; Ghosh et al., 2020; Hankey, 2020; Lim et al., 2012; Phan et al., 2019; Vujkovic-Cvijin et al., 2020). Several other factors associated with stroke, such as severity, reduced food intake post-admission, infection, underlying co-morbidities, and post-stroke medication and treatment, could also introduce significant variability in microbiota composition (Chamorro et al., 2007; FOOD Trial Collaboration, 2003; Vujkovic-Cvijin et al., 2020). Antibiotics certainly impact gut microbiota; however, current, or past usage (e.g., in the last two months) is included in the exclusion criteria of most if not all existing stroke-microbiota studies. Despite this, recent work has suggested that antibiotics may have a longer lasting impact on microbial composition, which should be taken into account (Vujkovic-Cvijin et al., 2020). At this stage, it is important to systematically collect metadata from patients to ensure that confounding variables can be controlled for (Michel et al., 2010). An overview of considerations during this stage and our recommendations are listed in Table 1. This is particularly important for human studies but also in animal models as well. It is well established that co-housing of mice homogenizes microbiota composition as mice are coprophagic, hence experimental findings must be replicated across multiple cages to ensure treatment/condition associated effects are reproducible (Robertson et al., 2019). Stroke specific factors also apply to animal models; mice subjected to severe stroke (transient occlusion of the middle cerebral artery) (Jackman et al., 2011) have a reduced food intake in the acute response (Lourbopoulos et al., 2016) in comparison to their sham counterparts, in part owing to sickness behavior (Roth et al., 2020), which likely impacts microbiota. To our knowledge, no stroke-microbiota study to date has accounted for this, however, controlling food intake, for example, by fasting sham mice, can mitigate this effect, improving the reliability of results. Stress induced by surgery and anesthesia also alter microbial composition, however using sham mice as a control likely reduces the impact of this (Singh et al., 2016). Nevertheless, it can be useful to include a non-surgical control in addition. Stroke severity itself (as quantified by infarct volume) also differentially impacts microbiota. Microbial composition of animals with small infarct volumes, induced by permanent middle cerebral artery occlusion (pMCAO) resemble sham mice (Singh et al., 2016). Infarct size also depends on the gender and age of experimental animals; thus, mice must be age and gender matched (Manwani et al., 2013). Examples of these considerations and our recommendations to mitigate them are compiled in Table 2.

Sample size

Estimating sample size is another often overlooked step, yet the validity and reproducibility of findings is highly dependent on this. Compounding this problem, very few software packages or tools currently exist to calculate sample size specifically for microbiota studies. Several R packages have been developed to solve this problem. Micropower, allows users to estimate the sample size required to detect differences in beta-diversity using PERMANOVA (Kelly et al., 2015). The recently published Powmic also enables power analysis for differential abundance testing (Chen, 2020). Despite this, only a limited number of microbiome

Table 2. Experimental design considerations for animal studies

Considerations	Recommendations
Microbiota standardization	Ensure external animals are acclimatized to facility for at least 1 week before experiment (Montonye et al., 2018)
Mouse congenital background and source	Comparison of microbiota composition between mice from same genetic background or same commercial vendors/facilities (Robertson et al., 2019)
Food	Standardization and sterilization (autoclave) of mouse diets
Litter and cage effect	Co-housing of animals of different treatment/conditions ensures microbiota shifts are not due to cage effect (Robertson et al., 2019)
	Randomization of experimental groups
	Replication of findings across multiple cages of different litters
Age and sex of mice	Use animals of similar age and the same sex, as both factors can impact stroke outcome (Manwani et al., 2013)
Timing of sample collection	Ensure samples are collected at approximately the same time of day to limit variation due to circadian rhythm (Liang et al., 2015)
Experimental model	Avoid direct comparisons of different experimental stroke models as some models impact microbiota composition more than others (Singh et al., 2016)
Food intake	Monitor weight loss and food intake after stroke/sham surgery by fasting of sham mice or weighing food
	Include weight loss as a covariate in analyses if significantly different between groups
Anesthesia	Record and standardize the dose, duration, and type of anesthesia given during surgical procedures

studies have utilized these tools (Debelius et al., 2016). In stroke, the severity appears to correlate with the magnitude of alterations in microbiota composition, thus when dealing with high variation in severity, this must be taken into consideration when deciding which samples to sequence (Singh et al., 2016; Xia et al., 2019; Yin et al., 2015). The microbiota also shows high variability for several weeks post-stroke (Xu et al., 2021). For longitudinal studies, this necessitates a larger number of total samples or utilizing multiple baseline samples to assess inherent variability. Well-planned and powered experiments and thoughtfully collected metadata underpin high quality studies as all downstream steps can be affected by choices made at this stage.

Sample type, collection, and storage

Owing to the relatively low cost and apparent ease of analysis, 16S rRNA sequencing is commonly used to profile microbial communities, providing a high-level, but low-resolution, overview of taxonomic composition. More in-depth methods such as shotgun metagenomics meta-transcriptomics and meta-proteomics provide information on both microbial composition and function but can be significantly more costly and require specialist resources to process and analyze data (Knight et al., 2018). Increasingly utilized in microbiome research, the chemical composition of the microbiome can also be measured via untargeted metabolomics (Bauermeister et al., 2021). Not all sample types are amenable to each method of profiling microbial communities; however, the choice of readout impacts the sample type that can be collected and dictates study design. In the stroke-microbiota field, there is currently a lack of diversity in sample types, particularly in humans, likely owing to logistical and ethical constraints. Results derived from stool samples are potentially limited in their translational impact as stool may not necessarily represent microbes involved in disease pathology (Claesson et al., 2017). Furthermore, stool samples provide a snapshot of the entire gastrointestinal tract but likely do not represent each compartment equally, reflecting distal gut sites better than proximal gut, which should be taken into consideration with respect to the scientific questions being asked. Here we focus on taxonomic profiling via 16S rRNA sequencing that, along with metabolomics and meta-proteomics, has the greatest breadth in terms of suitable sample types.

The method used for sample collection and subsequent storage can also introduce technical variation (Liang et al., 2020). Stool collection is the most used sample type, particularly in clinical studies and is likely

to be the most appropriate for human stroke-microbiota studies, owing to the difficulties of obtaining other relevant sample types. Multiple methods exist to sample stool; a specimen can be obtained directly or via rectal swab, yet results are conflicting on the impact of each on microbial composition. Rectal swabs, when processed quickly, reflect stool microbiota composition relatively well; however, Fair et al. have recently shown that rectal swabs may not accurately represent the microbiota of critically ill patients (Bokulich et al., 2019; Fair et al., 2019). As such, in stroke-microbiota studies, stool samples are preferred over rectal swabs, where possible.

In animal studies, there is often greater flexibility over sample type and collection; thus, the method chosen needs to be aligned with the scientific question. For example, profiling mucosal associated bacteria is more useful when studying the involvement of the microbiota in modulating intestinal immune response, post-stroke mucosal tissue impairment, and bacterial translocation (Houlden et al., 2016; Stanley et al., 2016). Several mucosal associated taxa, such as Segmented filamentous bacteria (SFB) in mice, induce intestinal T-cells under homeostatic conditions, as well as in stroke and are not reliably detected in stool (Goto et al., 2014; Sadler et al., 2017). Tissue and other ntestinal samples, such as cecal content, probably approximate local communities better than stool; however, obtaining these sample types requires sacrifice, restricting their use to cross-sectional studies. Regardless of the subject or sampling method, it is crucial to ensure samples are collected in the same manner and that comparisons between different collection methods are avoided.

Different sample storage conditions can additionally impact microbial abundances and composition (Chen et al., 2019; Liang et al., 2020; Marotz et al., 2021). In principle, extraction from fresh samples is best, however this is almost never feasible in practice. Ideally, samples should therefore be frozen at −80°C before extraction. For some study designs, this may present difficulties, particularly where samples are collected by patients themselves. For room temperature preservation, OMNIGENE Gut (Choo et al., 2015), 95% ethanol (Marotz et al., 2021), and FTA cards (Vogtmann et al., 2016) all suitably maintain composition during storage. As the most widely available and cost-effective method, we recommend storing samples in 95% ethanol at a ratio of 2:1 for stroke-microbiota studies where immediate storage at −80°C is not practical. As with sample collection, it is important to keep storage methods consistent between samples.

DNA extraction

Choice of DNA extraction kit can be another source of unwanted technical variation (Gerasimidis et al., 2016; Mackenzie et al., 2015). Various commercial kits are available, utilizing several methods of lysis – chemical, enzymatic, or mechanical – each significantly differing in resulting microbial composition. Gram positive and endospore forming bacteria are more resistant to lysis, particularly to chemical and enzymatic methods. Moreover, abundances of certain taxa also vary with the DNA extraction kit used. Mackenzie et al. compared five different commonly used extraction methods, showing that the abundance of Firmicutes and Bacteroidetes varied according to the method used (Mackenzie et al., 2015). Nevertheless, between-sample variation was greater than technical variation attributed to kit. Gerasimidis et al. reported similar findings and suggest that among the methods tested there was no one "best" method for all purposes (Gerasimidis et al., 2016). However, we recommend the use of a kit employing a mechanical-based lysis method. The inclusion of this step is associated with the increased quantity of microbial DNA, higher bacterial diversity, and increased recovery of Gram-positive taxa (Pollock et al., 2018). Ultimately, any approach needs to be optimized for sample type, have a high DNA yield, and not be biased toward any particular taxon.

Low-biomass samples such as biopsies present additional challenges owing to the presence of contamination in extraction reagents, recently termed the "kitome" (de Goffau et al., 2018; Nearing et al., 2021). Salter et al. elegantly highlighted this problem using a mock community dilution series, spiked with an unusual *Salmonella* strain (Salter et al., 2014). With increasing dilution, true biological reads were drowned out by contamination. Multiple studies have verified these findings, since finding that the kitome varies according to both batch and kit (Glassing et al., 2016; Weiss et al., 2014). For any study, we recommend a prudent use of negative extraction controls and to process all samples in one batch. Mock community controls can also be useful, particularly when optimizing methods. Negative and positive controls are essential for low biomass studies as contaminating bacterial DNA can mask true biological signal or lead to false conclusions (Salter et al., 2014). Bacterial DNA has been identified in many sterile sites

previously thought to be sterile, including the brain tissue. However, evidence for a "brain microbiome" is scant and additional work has been unable to separate contamination from true signal (Bedarf et al., 2021).

Library preparation and sequencing

Library preparation and sequencing are generally outsourced to a core facility or commercial sequencing provider, but the choice of hypervariable region(s) and primer pair – in the case of 16S sequencing – and sequencing platform is left to the researcher. As we are focused on amplicon sequencing of the bacterial component of the microbiome, other technologies are outside the scope of this review. Readers are referred to the following in-depth articles on metagenomics (Quince et al., 2017), meta-transcriptomics (Zhang et al., 2021), and metabolomics (Bauermeister et al., 2021). The 16S rRNA gene comprises nine hypervariable regions, V1–V9. The region and primer pair used will have a significant impact on both phylogenetic resolution and the taxa that are detected (Abellan-Schneyder et al., 2021; Chen et al., 2019). No existing primer pair are universal, and some pairs may miss biologically relevant taxa, for example, the commonly used 27f primer, amplifying the V1 region can miss some *Bifidobacteria* (Chen et al., 2019). Similarly, there is no gold standard hypervariable region, although V1–V2/V3, V3–4, and V4 are among the most commonly used regions in human and mouse microbiota studies that may aid comparison with existing published data (Abellan-Schneyder et al., 2021). Larger regions also increase the identification of certain taxa. The number of PCR cycles used to generate 16S amplicons will also affect microbial composition. A high number of cycles increases the possibility of bias, chimera formation and the impact of contamination (Sze and Schloss, 2019). However, this is generally only an issue for low biomass samples such as tissue biopsies, as the low input may require a higher number of cycles to produce sufficient input for sequencing, and pooling of PCR products can be used to mitigate this issue (Kennedy et al., 2014). The choice of primer pair and hypervariable region is mostly down to personal preference; however, for certain environments, some primer pairs are preferred. V1–V2 is recommended for oral microbiota, for example, as it produces the most faithful representation of oral communities (Wade and Prosdocimi, 2020). In any case, researchers should be aware of the pros and cons of each pair and choose accordingly.

The majority of 16S studies are sequenced on using Illumina MiSeq machines, owing to their high throughput, accuracy, and length of reads (up to 2 x 300 bp) (Caporaso et al., 2012). The chosen platform depends on the scientific question being asked but generally, MiSeq will be the most appropriate. Illumina HiSeq machines are also appropriate for both 16S and Shotgun metagenomics studies, offering higher accuracy at the expense of increased cost and shorter reads (Caporaso et al., 2012; Quince et al., 2017). However, recent newer Nextseq and Novaseq Illumina machines are being increasingly utilized in metagenomics, owing to reduced costs and similar output (Quince et al., 2017). In Illumina machines, reads can be sequenced in either single- or paired-end mode. We recommend opting for paired end as this can increase the coverage and amplicon length. Longer-read technologies such as Nanopore and PacBio sequencing are becoming more commonplace in recent years but traditionally suffered from high error rates and an underdeveloped bioinformatics ecosystem, encumbering analysis. However, this has improved vastly, with both Nanopore and PacBio sequencing achieving accuracies of >99.9% on full-length 16S sequences as of 2021 (Karst et al., 2021). Improvements to bioinformatics tools have also facilitated pre-processing and analysis but only a handful of microbiota analysis packages accept these kinds of data (Callahan et al., 2019). In the coming years, full-length 16S sequencing will likely be a viable alternative to short amplicon sequencing, and further enable the identification of taxonomic signatures in stroke and other diseases.

Bioinformatic tools for microbiota analysis

In this section, we outline methods and tools used for raw data processing and statistical analysis of microbiota data. As the application of these tools can require significant background knowledge, even those with well-designed microbiota studies can run into problems with their analyses. Here we provide an explanation of techniques and tools used and suggest various software packages and pipelines with extensive tutorials and documentation to enable researchers unfamiliar with the field to make informed choices. As microbiota analysis contains a substantial amount of field-specific jargon, we provide a glossary below explaining commonly used terms (Box 2).

Raw data and pre-processing

Raw **16S** rRNA sequencing data will generally be received in a fastq format, which requires substantial quality control and pre-processing before any inferences regarding microbiota composition can be

Box 2. Glossary

16S: The 16S rRNA small ribosomal subunit. Containing a combination of conserved and hypervariable regions it can be used as a molecular barcode to profile bacteria and archaea.
Alpha diversity: Species diversity within samples. Measures include **Richness**, **Chao1**, **Shannon,** and **Simpson** diversity.
ASV: Amplicon sequence variant. Error-corrected sequence representing the true biological sequence.
AUC (AUCROC): Area under the receiver operator curve. A measure used to assess the ability of a machine learning models to distinguish between classes.
Beta diversity: Between sample diversity. Metrics include **Bray-Curtis** and **UniFrac.**
Bray-Curtis: A count-based dissimilarity metric (**beta diversity**), based on the fraction of overabundant counts.
Chao1: An **alpha diversity** metric, which estimates species **richness** based on abundance of individuals belonging to a given class. Accounts for abundance and evenness.
Compositional data: Data composed of strictly positive numbers with a fixed sum (i.e., 1), conveying only relative information.
CLR transform: Centered-log ratio transform. A data transformation computed by calculating the log-ratio of each taxon relative to the geometric mean.
Cross-validation: A method of estimating the generalizability of a machine learning model by resampling limited data.
Differential abundance: Identification of bacteria that differ in abundance between groups.
Evenness: An **alpha diversity** metric, assessing how similar abundance distributions are between samples.
Faith'|'s phylogenetic diversity: A phylogenetic measure of **alpha diversity**, calculated as the sum of branch lengths in the phylogenetic tree.
False discovery rate (FDR): The proportion of findings that are falsely identified as significant.
NMDS: Non-metric multidimensional scaling. An ordination method that attempts to represent the dissimilarity between samples, as closely as possible in a low-dimensional space.
OTU: Operational taxonomic unit. A cluster of similar **16S** sequences (usually 97%) approximating a bacterial/archaeal genera or species.
PCoA (MDS): Principal coordinate analysis (also known as metric multidimensional scaling). An ordination method that attempts to preserve distance between samples in a low dimensional Euclidean space.
PERMANOVA: Non-parametric multivariate ANOVA. Used to assess whether beta diversity metrics differ between groups.
Machine learning: Use of data and algorithms to learn from data without explicit instruction. Machine learning models can be used to identify patterns in data and predict future trends.
Metagenomics: Measurement of the entire gene content present in a sample (DNA).
Metatranscriptomics: Measurement of actively transcribed genes in a sample (RNA).
Metabolomics: Measurement of the small molecule component (<2,000 Da) of a sample.
Shannon diversity: A measure of alpha diversity, accounting for both the number of observed species and species evenness.
Simpson diversity: An alpha diversity metric, measuring the relative abundance of species comprising sample richness.
Training/Test sets: Data splits used to train and test machine learning classifiers. The training set allows the model to learn patterns in the data while the test set's purpose is to estimate model performance on unseen data.
Rarefaction curve: A plot of species richness against read depth used to assess whether sampling depth was adequate. A plateau indicates that no further diversity is likely to be detected with increased depth.
Richness: An alpha diversity metric measuring the total number of observed species.
ROC: A visual aid to assess machine learning classifier performance where the true positive rate is plotted against the false positive rate.
UniFrac: A phylogenetic beta-diversity metric measuring the fraction of unique branches in a phylogenetic tree. The extensions weighted and generalized UniFrac also take relative abundance into account.

made. A multitude of pipelines and software packages are available, with varying levels of skill and technical knowledge required for use. Although pipelines with a graphical user interface (GUI) may seem like an attractive option, we maintain that it is better to invest the time to learn how to use more advanced and flexible tools, as this necessitates a greater understanding of the data. Widely used, well-maintained tools with extensive documentation such as QIIME2 (Bolyen et al., 2019), mothur (Schloss et al., 2009), and DADA2 (Callahan et al., 2016) are among some of the best options to pre-process and/or analyze microbiota data. A summary of all tools listed in this section and their use can be found in Box 3.

A crucial starting point in any analysis of sequencing data is the assessment of data quality (Figure 2, step 1). Tools such as FastQC and the extension package MultiQC enable an overview of sequence length, quality,

Box 3. Bioinformatic tools

Use	Tools	References
Quality Control	FastQC, MultiQC	(Andrews, 2010; Ewels et al., 2016)
Primer/adapter trimming	Cutadapt, Trimmomatic	(Bolger et al., 2014; Martin, 2011)
Amplicon denoising	DADA2, Deblur, UNOISE2	(Amir et al., 2017; Callahan et al., 2019; Edgar, 2016)
Taxonomy databases	RDP, SILVA, Greengenes	(Cole et al., 2013; DeSantis et al., 2006; Quast et al., 2012)
Phylogeny	FastTree	(Price et al., 2010)
Analysis suites	QIIME2, mothur, phyloseq	(Bolyen et al., 2019; McMurdie and Holmes, 2013; Schloss et al., 2009)
Pipelines	IMNGS, nf-core/ampliseq	(Lagkouvardos et al., 2016; Straub et al., 2020)
Differential abundance	ALDEx2, ANCOM/ANCOM-BC, DESeq2, gneiss, LEfSe, MaAsLin2, selbal	(Fernandes et al., 2014; Lin and Peddada, 2020b; Love et al., 2014; Mallick et al., 2021; Mandal et al., 2015; Morton et al., 2017; Rivera-Pinto et al., 2018; Segata et al., 2011)
Machine learning	mikropml, SIAMCAT	(Topçuoğlu et al., 2021; Wirbel et al., 2021)
Data repositories	ENA/SRA	(Leinonen et al., 2011)
Tools for reproducible research	GitHub/GitLab, Rmarkdown, Jupyter notebooks	(Allaire et al., 2021; Kluyver et al., 2016)

and adapter content (Andrews, 2010; Ewels et al., 2016). Low quality-reads, bases, and adapter sequences can then be removed using tools such as Cutadapt or Trimmomatic (Figure 2, step 2) (Bolger et al., 2014; Martin, 2011). Complete analysis suites such as QIIME2 and mothur wrap these programs or include their own scripts to perform these tasks. Low-quality or improperly filtered data can lead to various problems with downstream analyses, potentially inflating the diversity or hampering taxonomic classification.

Cleaned data can then be denoised, dereplicated, merged, if sequenced using paired reads, and then used to infer Amplicon sequence variants (**ASVs**) or alternatively clustered into 97% operational taxonomic units (**OTUs**) (Figure 2, steps 2 and 3). It is generally recommended to use **ASVs** as they increase the resolution and are comparable between experiments. DADA2 (Callahan et al., 2016), Deblur (Amir et al., 2017), and UNOISE2 (Edgar, 2016) are among the most widely used tools for **ASV** inference and all provide extensive documentation. **OTUs** are still a viable option for community-level analyses such as **alpha** and **beta diversity** and have been demonstrated to generate similar conclusions as **ASVs** from the same samples (Glassman and Martiny, 2018). Recent work utilizing mock communities recommends applying abundance filtering at 0.25% relative abundance to remove spurious **ASVs** or **OTUs**, as low abundant sequencing artifacts can impact downstream analyses, and we recommend including this step in any analysis (Reitmeier et al., 2021). After merging, using paired-end reads (Figure 2, step 4), **ASVs** or **OTUs** are assigned taxonomy, usually by a **machine learning** model trained on representative sequences (Wang et al., 2007). Most approaches use some variation of naive bayes by default, and, recently, Ziemski et al. demonstrated alternative models do not significantly improve on this (Figure 2, step 5). In this case, other methods should only be used with clear justification (Ziemski et al., 2021).

The choice of reference database, however, has a significant impact on the resulting taxonomic assignments. The Ribosomal database project (RDP) (Cole et al., 2013), SILVA (Quast et al., 2012), and Greengenes (DeSantis et al., 2006) are among the most comprehensive and most widely used reference databases. Using mock communities, Abellan-Schneyder et al. demonstrated considerable discordance in taxonomic assignment between databases, showing that RDP and SILVA performed consistently better than Greengenes (Abellan-Schneyder et al., 2021). Although there is no gold-standard database, we recommend using RDP or SILVA, as these are both larger and more recently updated than Greengenes.

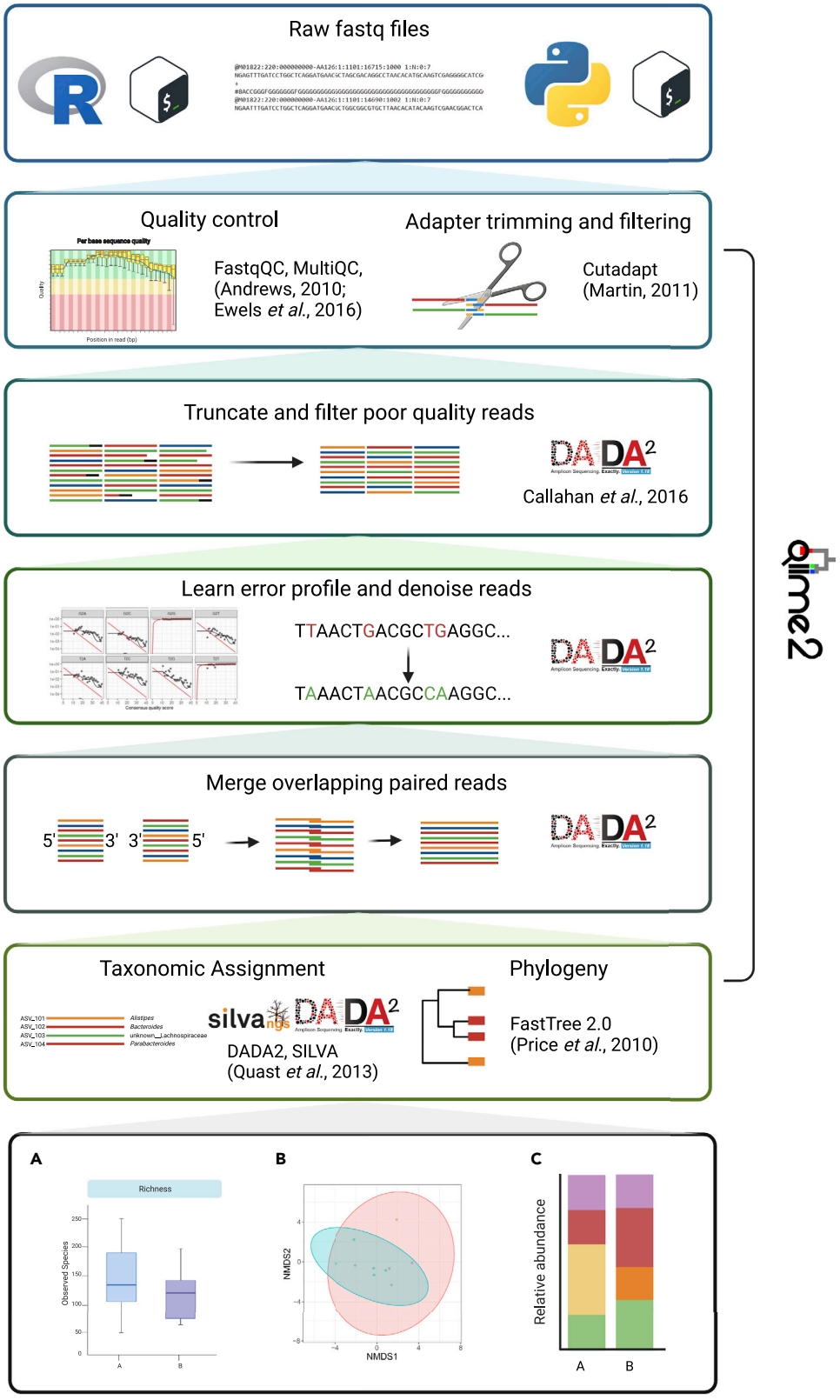
Raw fastq files
Quality control
FastqQC, MultiQC,
(Andrews, 2010;
Ewels et al., 2016)
Adapter trimming and filtering
Cutadapt
(Martin, 2011)
Truncate and filter poor quality reads
Callahan et al., 2016
Learn error profile and denoise reads
TTAACTGACGCTGAGGC...
TAAACTAACGCCAAGGC...
Merge overlapping paired reads
Taxonomic Assignment
DADA2, SILVA
(Quast et al., 2013)
Phylogeny
FastTree 2.0
(Price et al., 2010)
qiime2
A
Richness
Observed Species
B
NMDS1
NMDS2
C
Relative abundance

Figure 2. Data analysis pipeline

Overview of the data-analysis pipelines provided with this study, displaying each step in the analysis pipeline and the software used. Some examples of the kinds of figures which can generated with our pipeline are highlighted in the last step. Two versions of the same pipeline are provided, one written in R (left) and one in python via QIIME2 (right), which wraps the individual analysis steps in one software package (created with BioRender.com)

To generate a phylogenetic tree, **ASV** or **OTU** sequences are first aligned, and a tool like FastTree can be used to construct the phylogeny (Figure 2, step 5) (Price et al., 2010).

Each choice made during raw data processing will inevitably impact the data and it is critical that these choices are justified and well documented to enhance thee validity and reproducibility of findings. Using analysis pipelines in the form of scripts can help with this by providing a record of each step in the process. QIIME2 and containerized pipelines, such as the nextflow-based nf-core/ampliseq, extend this concept further by including provenance tracking and enabling fully reproducible analysis across various computing infrastructures in the case of nf-core (Bolyen et al., 2019; Straub et al., 2020). We recommend using such pipelines as they maintain the best balance between the ease of use and reproducibility.

Statistical analysis and interpretation

After **ASV** inference and taxonomic assignment, a matrix containing samples and the abundance of each unique taxon is generated. Microbiota data are generally high-dimensional, often containing thousands of **ASVs** and sparse, violating the assumptions of many common statistical tests. Additionally, each sample has differing read counts that must be managed before proceeding with any analysis. It is important to note that the number of reads is an additional important step in assessing the quality of the data. Defining a minimum threshold is difficult as different environments will vary in the number of reads recovered; however, work analyzing complex soil microbiomes has demonstrated even 2,000 reads per sample to be sufficient to capture the diversity of most samples (Caporaso et al., 2010). Plotting a **rarefaction curve** can also aid in assessing sampling effort.

To ensure measured differences are not an artifact of different read depth between samples, the data must be subjected to normalization before proceeding with downstream analyses.

Normalization is a contentious topic in the microbiota field, with no universally agreed-upon method. Rarefaction, which is randomly subsampling each sample to an even read depth, is very commonly used and yet has received criticism, as it discards valid data and reduces statistical power (McMurdie and Holmes, 2014). Scaling methods such as cumulative sum scaling or converting data into relative abundances avoid the problem of data loss but may lead to increased compositional bias and a higher **false discovery rate (FDR)**. Methods such as **centered log-ratio (clr) transform** mitigate **compositional** effects but are not compatible with many **alpha** and **beta diversity** metrics. As with many other steps in microbiome data processing and analysis, there is no gold standard, and readers are referred to the following publications that discuss the pros and cons of each in detail (Lin and Peddada, 2020a; McMurdie and Holmes, 2014; Weiss et al., 2017). The choice of normalization method should depend on the desired downstream analyses, with more advanced analyses potentially requiring a mixture of normalization strategies.

Assessing microbial community structure

After normalization, variations in the community-level structure can be assessed using alpha and beta diversity metrics. **Alpha diversity** measures diversity within samples. Commonly used metrics such as **richness**, **Shannon index** and **Faith's phylogenetic diversity (Faith's PD)** measure different aspects and can be used complementary to each other (Finotello et al., 2016). **Richness** is a simple measure of the number of observed species, whereas the **Shannon index** takes both species **richness** and **evenness** into account. **Faith's PD** integrates phylogenetic information to estimate species diversity. Other metrics such as **Chao1** and **Simpson index** are also often used; however, they are analogous to **richness** and **Shannon,** respectively, and are redundant if used in combination (Finotello et al., 2016). **Beta diversity**, on the other hand, measures the pairwise dissimilarity between samples and is used to assess how similar or different two given microbial communities are. The choice of metric can significantly influence the results obtained; thus, the metric used should be justified by the biological question. **Bray-Curtis**, a counts-based metric, measures the compositional dissimilarity between sites/groups while phylogenetic methods, such as **UniFrac,** take the genetic relationship between species into account (Lozupone and Knight, 2005).

Weighted and **Generalized UniFrac** are extensions of this method, which also account for abundance into, differing in terms of their sensitivity to lowly and highly abundant lineages (Chen et al., 2012; Lozupone et al., 2007). Differences in **beta diversity** can be visualized using ordination methods like **principal coordinates analysis** (**PCoA**; also known as **multidimensional scaling**) or **mon-metric multidimensional scaling (NMDS)**. Both **NMDS** and **PCoA** are suitable for all commonly used metrics; however, **PCoA** can generally only detect linear trends, whereas **NMDS** additionally detects non-linear patterns. Additionally, using **PCoA** with non-Euclidean distance metrics such as **Bray-Curtis** can produce negative eigenvalues, which cannot be represented meaningfully, potentially hindering interpretation. In this case, additional corrections must be applied before plotting. Researchers should be aware of the basics of each **beta diversity** metric and choose an appropriate ordination method accordingly.

Differential abundance

Beyond community-level changes, a common goal is to identify taxa that are associated with disease or differentially abundant between sample groupings. A recent meta-analysis of human microbiota studies found that among the 224 publications included 34 different methods of **differential abundance (DA)** were used, with LEfSe being the most common (Bardenhorst et al., 2021; Segata et al., 2011). This magnitude of different options can seem overwhelming, often leading to researchers choosing sub-optimal methods. Methods developed for RNAseq data such as DESeq2 have also been widely applied to microbiota data, which have been shown to perform poorly (Lin and Peddada, 2020a; Love et al., 2014), in part owing to not accounting for data compositionality. Although there is still some debate over whether microbiota data are truly compositional, compositionally aware methods are becoming more widely used, leading to the development of **DA** tools accounting for this (Gloor et al., 2017; Lovell et al., 2020). **Compositional data** refer to data that sum to a fixed constant e.g., 1 or 100, as with relative abundances. As these data are proportional, an increase in the abundance of one species necessitates a decrease in others, which can be misinterpreted by non-compositional methods. Novel methods such as ANCOM – including its recent extension ANCOM-BC – and ALDEx2, account for the compositional nature of microbiota data through the use of log-ratios, which makes the data scale invariant (Fernandes et al., 2014; Lin and Peddada, 2020b; Mandal et al., 2015). Other approaches such as selbal and gneiss utilize a concept known as "Balances," whereby the log-ratios of groups of taxa rather than individuals are associated with a response variable, enabling compositionally-aware identification of microbial signatures (Morton et al., 2017; Rivera-Pinto et al., 2018). We recommend using compositional methods as they are generally more conservative, reducing the potential number of false positives. Regardless of the **DA** method used, controlling the **FDR** is a crucial step to ensure that the number of false positives is limited. Kleine Bardenhorst et al. found almost half included studies did not correct for multiple-testing (Bardenhorst et al., 2021). Owing to the high dimensionality of microbiota data, significant results can be expected just by chance; therefore, it is essential to use an appropriate method to control type I error. Most **DA** packages include this by default but researchers should always be aware of the problem of multiple-testing and ensure the **FDR** is adequately controlled for. The impact of multiple testing can also be mitigated by including an additional abundance and/or prevalence filtering step prior to **DA** testing, removing taxa below a given threshold. For example, in our laboratory, we commonly use a prevalence filter, retaining taxa present in more than a third of samples; however, we recommend defining thresholds operationally as, in some cases or environments, low abundant taxa may be relevant to outcome.

Machine learning for the prediction of disease-associated microbiota

Machine learning (ML) is a promising method to identify potential microbial biomarkers associated with disease but is perhaps the most abused analysis technique in microbiota research. Many studies report extremely high classification accuracies, using metrics such as **Area under the receiver operator curve (AUC)**, but very few report low performance. The complexity and high variability of microbiota data would imply that this is likely to arise from other factors rather than the true biological signal (Quinn, 2021). Many microbiota studies have relatively low sample sizes or imbalanced groups, which, when combined with the large number of features (i.e., **ASVs** or **OTUs**), present significant difficulties when training **ML** models, leading to overfitting (Teschendorff, 2019). The minimum sample size to use depends on the complexity of both the given data set and the model used. As a rule of thumb, we do not recommend training models on data sets with a sample size <40 (Wirbel et al., 2021). Compounding this issue, small sample sizes can often lead to researchers omitting **test sets**, artificially inflating performance and leading to unverified results. Another common mistake is **test set** leakage, where information from the **test set** is included in the **training set**, which can similarly inflate model accuracy. Estimates of accuracy can be obtained by using **cross-validation**, where the data set is repeatedly split into **training and test sets** and the accuracy

averaged across each fold. However, in many cases, this is used incorrectly, also often owing to **test set** leakage, or not at all (Quinn, 2021). Finally, supervised feature selection – that is, preselecting features that are differentially abundant or show high feature importance – is often performed before training an **ML** model, for example, by filtering data sets to only include differentially abundant taxa, again leading to overly optimistic model accuracy (Wirbel et al., 2021). Feature selection should be nested within the **cross-validation** process to ensure reliable estimates of model accuracy.

Clinical stroke-microbiota studies using of **ML** have generally demonstrated acceptable use of **cross-validation** and **test** and **training sets**, however many have performed supervised feature selection (Li et al., 2019; Xu et al., 2019; Yin et al., 2015). Together, these issues hamper identification of accurate microbial signatures of disease. The correct use of **ML** models and larger sample sizes will allow the identification of robust bacterial signatures of stroke, as already demonstrated for many other diseases (Asnicar et al., 2021a; Ghosh et al., 2020). To ensure the correct use of **ML**, researchers can make use of R packages like mikropml and SIAMCAT that automate most of the steps where researchers unfamiliar with **ML** can make mistakes (Topçuoğlu et al., 2021; Wirbel et al., 2021).

Longitudinal analysis of microbiota data

Longitudinal sampling designs enable additional insights, enabling concurrent analysis of both inter and intra-individual variability, however in stroke, temporal analyses have been limited thus far, yet are likely crucial in identifying microbes involved in modulating outcome. Such designs present additional analysis challenges, owing to the inherent autocorrelation of temporal data, which violates the assumptions of many common statistical tests. Care must therefore be taken to ensure the correct tests are used, particularly for **DA** or **ML** analyses.

Microbiome-specific methods or packages for time-series analysis are currently scarce; however, recent versions of QIIME2 ship with a plugin for longitudinal analysis: q2-longitudinal that utilizes various methods to measure the temporal stability and identify **DA** features (Bokulich et al., 2018). Most of the existing **DA** methods mentioned above are either not suitable at all for temporal data, or not suitable without modifying default parameters. ALDEx2, ANCOM-BC, and MaAsLin2, however, all implement models that can be used to perform longitudinal **DA** (Fernandes et al., 2014; Lin and Peddada, 2020b; Mallick et al., 2021). For a more detailed review and tutorial on analysis of time-series data, we refer the readers to Coenen et al. (2020).

Controlling for confounding variables

Confounding variables can lead to false identification of differentially abundant taxa or exaggerate **AUC**, in the case of **ML**. Unfortunately, confounders are plentiful in microbiome research, which can make it difficult to discern true signal from noise (Vujkovic-Cvijin et al., 2020). As discussed above, it is important to collect as much detailed and relevant metadata as possible as this can be extremely helpful at this step in the analysis. We recommend always plotting data before beginning any further analyses. For example, when conducting **alpha** or **beta diversity** analyses, it can be informative to generate **PCoA/NMDS** plots colored by additional variables than those of direct relevance to the scientific question of the study, to assess the potential impact of confounding variables. However, this will only highlight confounders thatcontribute significantly to variation in the data. In some cases, careful grouping of samples, or, dependent on sample size, exploring subsets of groups can mitigate the impact of confounding variables. We recommend using packages where confounders can be included as interactions, such as gneiss, ANCOM-BC, or MaAsLin2 (Lin and Peddada, 2020b; Mallick et al., 2021; Morton et al., 2017).

Fostering reproducibility and transparency

To ensure the reproducibility and validation of the findings, raw data and associated metadata need to be openly accessible before publication via public repositories such as the Sequence read archive/European Nucleotide archive (SRA/ENA) (Leinonen et al., 2011). At the very least, every software package and the specific version used need to be listed in the methods of any resulting manuscript. In the case of automated pipelines, this information should be sufficient to reproduce the analysis. However, for bespoke analyses, pipeline scripts used to process the raw data should be shared in a publicly available repository such as GitHub or GitLab. Documenting each step in the analysis process is also important and further fosters reproducibility and transparency. Various tools are available to aid in this process and we strongly recommend making full use of these. Writing code in Rmarkdown or Jupyter Notebooks/Jupyter Lab allows the inclusion of text in markdown format between cells of code, facilitating thorough documentation of the

analyses performed (Allaire et al., 2021; Kluyver et al., 2016). Using a version control software, such as Git or Mercurial, is also encouraged and enables both researchers and collaborators to better track analyses. Similarly, a analysis code should also be uploaded to a public repository after publication. Ensuring clear and detailed reporting will boost the stroke-microbiota field, facilitating novel insights into the interaction between gut bacteria and stroke. Moreover, consistent and rigorous standards will enable meta-analyses allowing identification of microbial signatures of stroke and stroke outcome.

Best practices – example pipeline

Theory and recommendations are necessary to help guide stroke-microbiota researchers toward making the correct decisions regarding their data; however, practical advice and tutorials for some steps are often lacking. To this end, we have designed and conducted our own stroke-microbiota study in mice, investigating differentially abundant taxa, so that other researchers can use this as a template for their own studies.

Here, we designed a simple experiment to identify enriched taxa in mice subjected to experimental stroke. Mice were randomly split into two groups, and either sham surgery or transient middle cerebral artery occlusion (tMCAO) was performed (Llovera et al., 2021). Stool samples were collected 3 days after surgery and frozen at −80°C before extraction and subsequent sequencing of the V4 region of the 16S rRNA gene.

To enable a wider number of researchers to utilize our pipeline, we have provided two versions of our analysis pipeline, one for those familiar with R and another for those more familiar with python. Each step in the analysis pipeline is summarized above (Figure 2).

Results summary

To confirm the presence of ischemic stroke, we performed cresyl violet staining of brains from mice subjected to tMCAO. All mice developed moderately large lesions in the ipsilateral hemisphere (mean 40.6 $\pm$ 12.6 mm^3; Figure 3A). Previous work comparing the microbiota of stroke and sham mice has identified broad changes in microbial community structure but a limited loss of sample diversity. This suggests that modulation of stroke and stroke outcome by the microbiota is mediated by changes in microbial composition, rather than a loss of taxa. Thus, to confirm the absence of changes in overall diversity and evenness, we calculated richness, Shannon effective, and Faith's PD assessing the observed number of species, diversity, and evenness and phylogenetic diversity. No significant differences were observed between sham and stroke mice using any alpha diversity metric (Figure 3B), which is in line with previous findings from murine studies (Houlden et al., 2016; Singh et al., 2016).

We next sought to assess differences in global community structure between sham and stroke mice. Unsupervised analysis based on Generalized UniFrac distance revealed stroke samples clearly separated from controls. Consistent with prior studies showing a differential impact of stroke severity on gut microbiota, mice with larger infarcts tended to be further from sham mice than those with less severe strokes (Figure 3C). To visualize taxonomic changes driving this difference, we plotted the taxonomic composition of each sample by experimental group. It is important to note that the conclusions made from these types of visualizations are generally limited; however, they can provide a useful overview of potentially interesting changes in relative abundance. Shifts in relative abundance at the family level in stroke appeared relatively minor, implying microbial shifts occur at lower taxonomic ranks (Figure 3D). Finally, we sought to investigate changes in microbial abundance in detail. Using ANCOM-BC, a compositional DA method, we identified five ASVs with a corrected p-value < 0.05, in stroke mice (Figure 3E). These were classified as *Blautia*, unknown Lachnospiraceae, *Parabacteroides*, unknown Rikenellaceae and *Alistipes*. We additionally identified 17 ASVs decreased in stroke mice, several of which are known butyrate producers, previously identified as decreased in stroke patients (Haak et al., 2021).

Concluding remarks

Here we have provided a blueprint for conducting stroke-microbiota studies along with an example data set recapitulating and extending current findings. Adherence to these guidelines will enable further insights on the involvement of the microbiota in stroke, facilitating targeted manipulation, with the potential to improve stroke outcomes. Beyond 16S rRNA sequencing, we encourage further adoption of other technologies such as shotgun metagenomics, meta-transcriptomics, and metabolomics/meta-proteomics coupled with multi-omic integration in stroke-microbiota research. Multi-omic integration of these different data types aids in functional understanding of microbial factors influencing a disease.

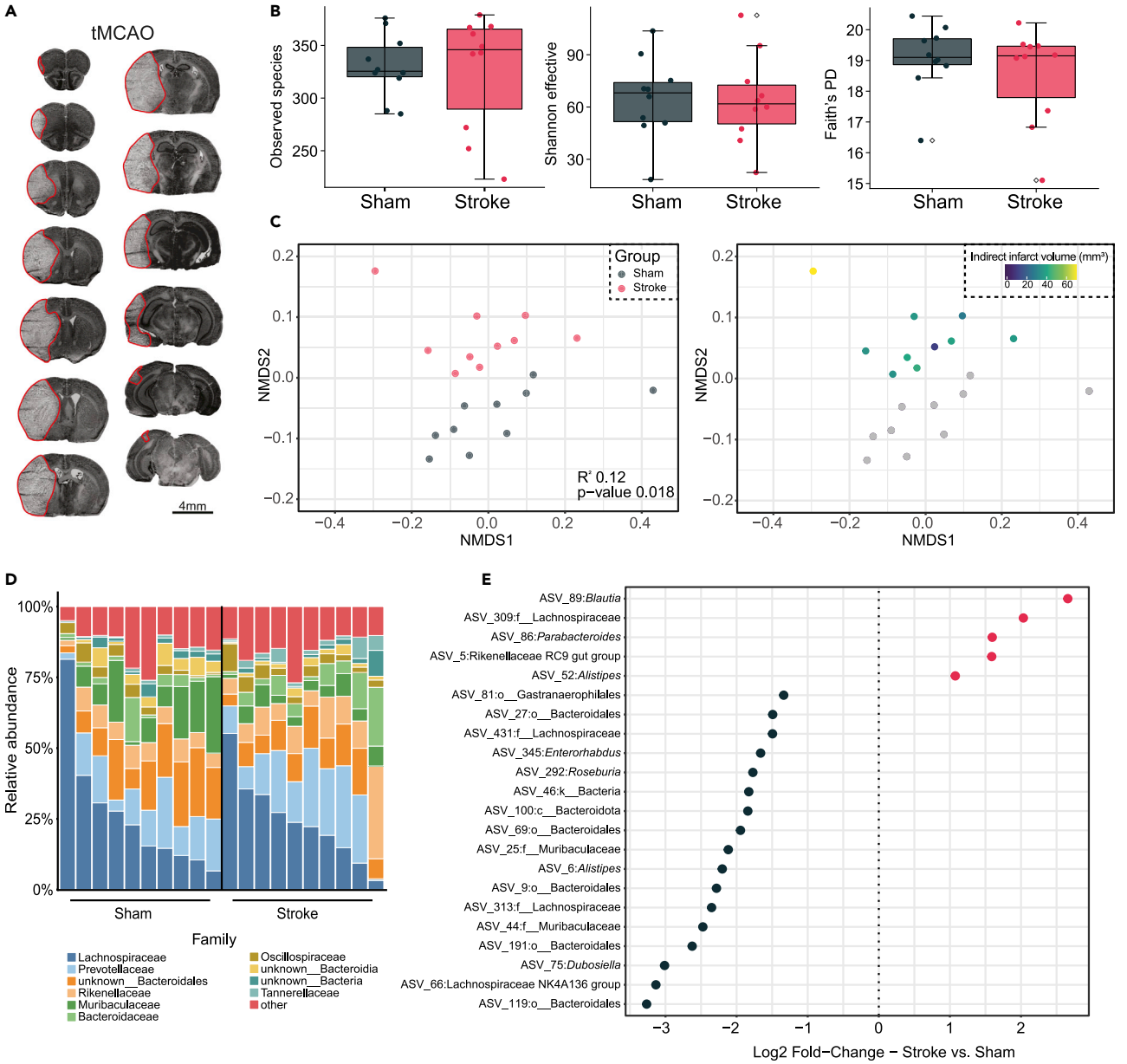

Figure 3. Disruption of community structure and composition post-stroke

(A) Representative cresyl violet stained sections, 3 days after tMCAO. Scale bar: 4 mm

(B) Alpha diversity measurements between stroke and sham mice showing richness (left), Shannon effective (center), and Faith's phylogenetic diversity (right). Individual points dots represent a single mouse. Error bars represent the median +/− 1.5 multiplied by the IQR. Outliers are highlighted by an empty square

(C) Non-metric multidimensional scaling plot of Generalized UniFrac distance colored by group (Adonis PERMANOVA R^2 = 0.12, *p*-value = 0.018)

(D) Family-level relative abundance in sham and stroke mice. Low abundant families were grouped with each other

(E) Significant differentially abundant taxa between stroke and sham mice, identified by ANCOM-BC (corrected *p*-value <0.05). Log2 Fold-change between conditions is shown on the *x* axis

Additionally, reductionist approaches such as gnotobiotic mice can help identify individual microbes that can alter a disease or disease course. Employing these technologies in the stroke-microbiota field will further mechanistic insights into the interplay between stroke and the microbiota.

We hope that this manuscript will lead to an improvement in the consistency, transparency, and reproducibility of methods and believe this is required before consistent microbial signatures can be identified,

laying the groundwork for microbiota-based treatments to become a reality. This, combined with the adoption of methods to delineate functional mechanisms, will enable the translation of stroke-microbiota research into clinical practice.

Limitations of the study

The limitations of this study include the focus on 16S rRNA sequencing at the expense of other, more detailed, profiling techniques such as shotgun metagenomics. In addition, our data were collected at a single timepoint, which may miss important microbial shifts associated with stroke that a longitudinal sampling design would uncover.

STAR★METHODS

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AUTHOR CONTRIBUTIONS

A.S.: conceptualization, data curation, formal analysis, investigation, methodology, supervision, visualization, writing original draft; R.D.: investigation, methodology; C.B.: funding acquisition, supervision, project administration, conceptualization, manuscript review, and editing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
DNeasy PowerLyzer PowerSoil Kit	QIAGEN	Cat. No. / ID: 12855-50
Deposited data		
Raw sequencing data	This publication	ENA: PRJEB48735
Processed data and metadata	This publication	https://github.com/adamsorbie/Stroke_Microbiota_reproducibility/tree/main/data
Software and algorithms		
Powmic	(Chen, 2020)	https://github.com/lichen-lab/powmic
FastQC/MultiQC	(Andrews, 2010; Ewels et al., 2016)	https://github.com/s-andrews/FastQC/ https://multiqc.info/
Cutadapt	(Martin, 2011)	https://cutadapt.readthedocs.io/en/stable/
DADA2	(Callahan et al., 2016)	https://benjjneb.github.io/dada2/index.html
QIIME2	(Bolyen et al., 2019)	https://qiime2.org/
Phyloseq	(McMurdie and Holmes, 2013)	https://joey711.github.io/phyloseq/
R	The R foundation	https://www.r-project.org/
ImageJ	N/A	https://imagej.nih.gov/ij/
BioRender	BioRender	https://biorender.com/
Other		
Chow	Ssniff	V1574-300

RESOURCE AVAILABILITY

Lead contact

Further information and requests should be directed to and will be fulfilled by the lead contact, Corinne Benakis (Corinne.Benakis@med.uni-muenchen.de).

Material availability

This study did not generate new unique reagents.

Data and code availability

- Raw sequencing data were deposited in the European Nucleotide Archive under project number: PRJEB48735 and are publicly available as of the date of publication.
- Scripts used for data processing and analysis are publicly available on GitHub and are publicly available at the following url: https://github.com/adamsorbie/Stroke_Microbiota_reproducibility.
- Any additional information required, is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All experimental protocols were approved by the responsible governmental department (Regierung von Oberbayern, Munich, Germany). C57BL/6 male mice were purchased from Charles-River Laboratories (Sulzfeld, Germany), and acclimatized for 1 week. Mice were fed a standard chow diet (Ssniff, Soest, Germany) fed *ad libitum* and housed under SPF conditions (12 h light-dark cycles at 22 $\pm$ 2°C). Mice were co-housed until surgery and subsequently placed in separate cages for 72h to allow food intake and weight loss to be monitored.

Middle cerebral artery occlusion

Transient middle cerebral artery occlusion (tMCAO) was induced in 8-12 week old mice as previously described with monitoring cerebral blood flow by transcranial laser Doppler flowmetry (Jackman et al., 2011; Llovera et al., 2021). Briefly, mice were anesthetized with 1.5–2% isoflurane, and rectal temperature maintained at 37 ± 0.5°C for the duration of surgery. A silicon-coated filament (#602112PK5Re, Doccol) was inserted into the left external carotid artery and advanced until obstructing the MCA together with ligation of the common carotid artery for 45 min. Regional cerebral blood flow (CBF, bregma coordinates: 2-mm posterior, 5-mm lateral) was recorded by transcranial laser Doppler flowmetry (PF 5010 LDPM, Periflux System 5000, Perimed) at induction of ischemia, before filament removal and at reperfusion. When MCA occlusion was confirmed (a residual CBF <20%) animals were placed in a recovery chamber at 37°C for 45 min (until filament removal). To allow reperfusion, mice were anesthetized after occlusion and the filament was removed. Mice were included if the residual CBF is <20% before reperfusion and CBF recovery >80% within 10 min of reperfusion. Following tMCAO, mice were placed in temperature-controlled recovery cages for 2 h to prevent post-surgery hypothermia. Sham surgery was performed as described above including ligation of the external and common arteries, and introduction of the filament (in/out). At 72h after surgery, mice were euthanized, brains were collected and immediately frozen at –80°C for cresyl violet staining.

METHOD DETAILS

Infarct volume quantification

Brains tissues were sectioned (30-µm thick at 600-µm intervals) on a cryotome for cresyl violet staining as previously described (Benakis et al., 2020). Quantification of the infarct volume (corrected for swelling and tissue loss) was performed blinded using ImageJ.

Power analysis

Sample size estimation to detect differentially abundant taxa was carried out using powmic (Chen, 2020). Parameters were estimated by fitting a subset of a previously published dataset (Xu et al., 2021), selected to closely mirror our own study design, to a zero-inflated negative binomial model. These parameters were subsequently used to generate synthetic datasets with 1000 ASVs. Estimation of the false discovery rate (FDR) and the true and false-positive rates was then performed using the powmic function with default parameters, testing a range of sample sizes (six, eight, ten and twelve). We used a true-positive rate threshold of 0.8 to determine sufficient power.

DNA extraction and 16S sequencing

DNA was extracted from fecal samples using a QIAGEN DNeasy Powersoil Kit and quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher, United States). Sequencing was performed by the Beijing Genomics Institute (BGI) according to standard procedures. Briefly, the V4 region of the 16S rRNA gene was amplified using the 515F- GTGCCAGCMGCCGCGGTAA and 806R-GGACTACHVGGG TWTCTAAT primer pair and sequenced on an Illumina HiSeq™ 2500 machine using 2x250 bp paired end reads.

Quality of raw reads were checked with MultiQC (Ewels et al., 2016) before trimming with Cutadapt (Martin, 2011). Subsequently, ASVs were inferred using DADA2, and filtered using a threshold of 0.25% relative abundance to remove spurious sequences (Reitmeier et al., 2021). The remaining ASVs were assigned taxonomy using the SILVA database version 138 (Callahan et al., 2016; Quast et al., 2012) and a phylogenetic tree inferred using FastTree2 with the GTR+CAT model (Price et al., 2010). Steps were identical in the python-based pipeline, except that QIIME2 was used, which wraps the aforementioned tools.

QUANTIFICATION AND STATISTICAL ANALYSIS

Alpha (richness and Faith's PD) and beta diversity (Generalized UniFrac) were calculated using R or QIIME2 (Bolyen et al., 2019). Statistical analysis of alpha and beta diversity was performed using Wilcoxon-tests and PERMANOVA respectively. Differential abundance analysis was performed using ANCOM-BC (Lin and Peddada, 2020a) using the Benjamini Hochberg method to control the FDR.