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Best practices framework for using 16S rRNA gene sequencing in poultry microbiota research

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ABSTRACT

Microbiome research has shown significant potential in enhancing poultry health and productivity. Despite the increasing volume of data linking the microbiota with various host traits, challenges persist regarding the consistency and reproducibility of findings. A major underlying issue is the variability in methodologies employed across studies. As such, a need exists to establish a set of standardized guidelines that help guide experimental design, DNA extraction, sequencing, data analysis, and reporting in poultry microbiota research. Rather than advocating for a single standardized protocol, we propose a best practices framework designed to enhance methodological rigor while accommodating distinct research contexts. Such a framework emphasizes the use of appropriate positive and negative controls and improved data reporting to facilitate cross-study comparisons and reproducibility. As a companion to our previous review of the 16S rRNA gene sequencing landscape in poultry microbiota research, this manuscript represents a collaborative effort among experts from academia, industry, and government. It aims to offer a practical set of guidelines that serve as a checklist for designing, conducting, analyzing, and reporting poultry microbiota studies. These guidelines seek to improve consistency, reproducibility, and robustness of poultry microbiota research.

Introduction

Over the last two decades, scientific interest in the poultry gastrointestinal tract (**GIT**) microbiota has skyrocketed, with the microbiota being linked to virtually every aspect of poultry health and production. This relatively recent surge of interest in the poultry

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microbiota, which herein refers explicitly to the community of bacteria, is in part a product of the development, low cost, and ease of access to next-generation sequencing (**NGS**) technologies. Although NGS platforms have enabled unprecedented insight into the bacterial communities that inhabit the poultry GIT, little has been done to address issues that exist concerning consistency, reproducibility, and robustness of microbiota findings in poultry. As both industry and academic interest in the poultry microbiota is continuously increasing, there is an urgent need to develop a set of guidelines that establishes a best practice approach for conducting poultry microbiota research. Our aim here is not to set forth strict criteria to be rigidly followed in every poultry microbiota study, but to provide the researcher with an evidence-based roadmap that will aid in the design, implementation, and analyses of reproducible poultry microbiota research.

At the outset, it is important to take a brief historical perspective of poultry microbiota research and understand why, despite the last decade seeing a record number of publications associating the microbiota with avian physiology, a best practice approach in this field of research is now needed. Since the early 20th century, germ-free poultry (i.e. birds that have no microorganisms) have been used to demonstrate the immense impact that the microbiota has on avian physiology (Cowieson, 2022; Lee et al., 2022). In these foundational studies, germ-free birds and the role of the microbiota were largely explored through microbiological means (Cole and Boyd, 1967; Forbes et al., 1959; Morishita and Mitsuoka, 1976; Phillips et al., 1962; Salter and Coates, 1971; Salter and Fulford, 1974; Siddons and Coates, 1972). With the advent of second-generation sequencing technologies (e.g. pyrosequencing) (Callaway et al., 2009), and later third-generation sequencing technologies (Heather and Chain, 2016), culture-based methods began to compete with NGS to study the microbiota along the GIT in poultry (Thomas et al., 2019).

The expertise and skillsets required for investigators to study the poultry microbiota have shifted from the use of conventional microbiological techniques to the use of next-generation sequencing technologies and bioinformatics. This is not to say culture-based methods have been replaced, quite the contrary, more conventional microbiology and culture-based methods should be utilized in modern day microbiota research. Instead that what was once the domain of conventional microbiology is now largely integrated with bioinformatics (Hiergeist et al., 2015). Poultry researchers in pursuit of studying the microbiota now confront a catalog of DNA extraction kits, tissue storage options and reagents, library preparation kits and methods, sequencing platforms, software tools, and reference databases.

Indeed, the overwhelming number of tools available to researchers create a monumental number of different paths to go from design to data analysis. Despite the expansion of technologies being beneficial to researchers, the lack of standardization creates inconsistencies among microbiota studies; thereby, creating difficulty in the meaningful comparison of results between studies which, in turn, impedes the application of microbiota-based approaches to improve poultry production (Applegate et al., 2010). Awareness of the need for standardization in poultry microbiota research has been recently highlighted in poultry-focused work (Lyte et al., 2025; Marková et al., 2024).

As such, it is our objective that the present manuscript serves the poultry science community as an evidence-based set of best practice guidelines that enable reproducibility and consistency in poultry microbiota research.

Experimental design

Study design is a critical determinant of the validity and reproducibility of microbiome research. As microbiota studies deal with many unknown or hidden host and environmental variables, the choice of study design can have a great impact on study outcomes and data interpretation. Providing details on a broad range of host and environmental factors, in publications and sequence data repositories is highly recommended and creates opportunities to combine data from multiple studies for meta-analysis, which will then facilitate scientific breakthroughs toward nutritional and husbandry-associated strategies to improve animal health and performance (Kers et al., 2018).

The statistical analysis performed depends on the study design (Fig. 1), and the type of data collected. Given high inter-individual variability in microbial communities, stratified sampling and adequate replication are essential. In addition, standardized protocols for sample collection, DNA extraction, sequencing, and bioinformatics are important to minimize technical biases within a study. Technical variation might result in batch effects and need to be considered to avoid the risk of losing true biological differences.

Longitudinal designs can provide insight into microbial dynamics over time, while cross-sectional studies offer snapshots of community structure under varying conditions. Clear hypotheses, appropriate positive and negative controls (e.g., mock community, and an empty sample collection tube), and integration of a wide variety of host and environmental data further strengthen microbiome study designs, enabling a more comprehensive understanding of microbial roles in health, disease, and ecological function.

Poultry host-related variables include breed, age of parent flock, maternal factors, age of the sampled bird, sex, body weight, health status, and performance indicators such as feed conversion ratio. Environmental and management factors such as housing system (e.g., cage, barn, or free-range), stocking density, light schedule, litter type and condition, temperature, humidity, ventilation, vaccination program and schedule, and biosecurity measures can significantly influence microbial community structure and must be carefully documented (Fig. 1). Another important factor is feed data, including composition, use of additives or medications (e.g., prebiotics, probiotics, postbiotics, antibiotics, enzymes, among others), and feeding schedules, as diet is a major modulator of gut microbiota (Olson et al., 2022). Feed microbial composition such as the presence of *Salmonella* in the feed may also be a contributing factor to bird GIT development (Olson et al., 2022). Moreover, information regarding physicochemical properties of feed, including particle size, ingredient batch and geographical origin, along with storage time of ingredients before allocation to poultry have demonstrable impact on the microbiome (Novotný et al., 2023; Rubio-Cervantes et al., 2026; Stanley and Bajagai, 2022), and therefore should be considered in the experimental design and reported in the methodology or metadata.

Metadata

Metadata corresponds to all information related to the sample other than the identification of the sample itself. It means that metadata contains the informative and relevant description about the original sample or sets of samples. It helps putting context around all data collected of a sample and connecting it to other datasets and information. Metadata helps the reader to select which information is considered important and allows searches across larger databases for comparison. Metadata also contributes to clarify trial material and methods so others would know how to reproduce the study design. In the case of a poultry sample taken for microbiome analysis different levels of information can be collected.

Sample identification

Samples are frequently identified by unique codes that help avoid mistakes during sample collection, storage, processing, and data analysis. The identification usually includes trial code, treatment, pen number, bird ID, age, sex, and sample type. This sample identification should always be unique within and among datasets.

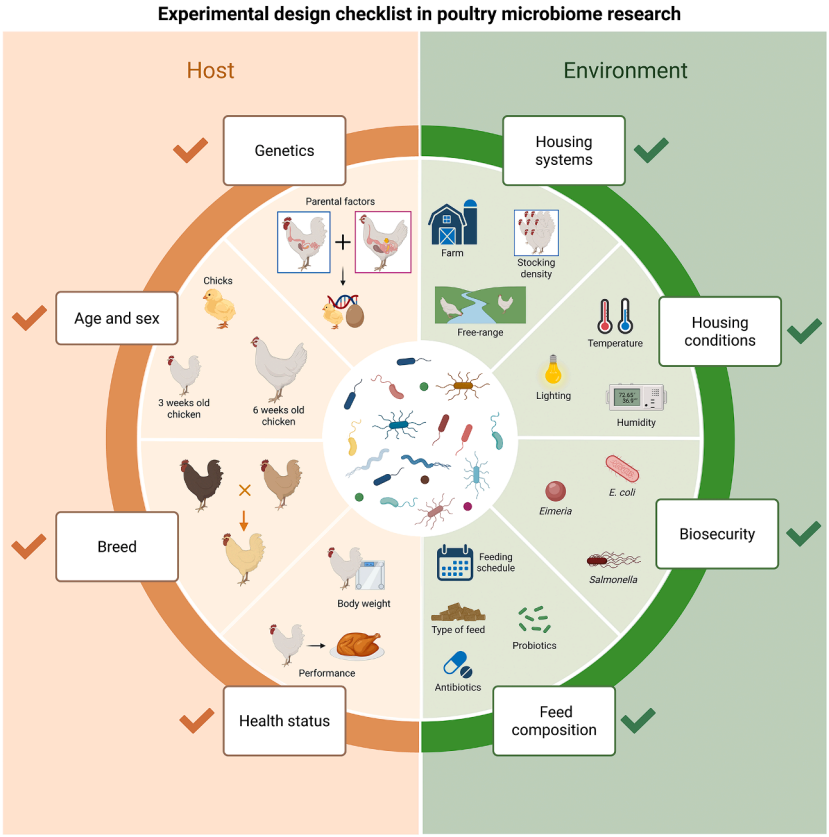

Fig. 1. Experimental design checklist in poultry microbiome research. Schematic representation of host-and environment-related factors to document when performing 16S rRNA gene sequencing of the poultry intestinal microbiota. Created with BioRender.com.

Sample metadata

On top of the information used to uniquely identify a sample, it is important to associate other kinds and levels of data related to a certain sample. Part of the metadata may be unique at the trial level, meaning the same information applies to all samples in that dataset or trial but

Sample ID			
Trial code	**Tube number**		
Pen			
Block			
Treat code			
General (trial level)			
Trial code	**Trial type (controlled, field study)**	**Feed additives**	**Feeding schedule**
Country	**Parent breeder age**	**Vaccination**	**Vaccination schedule**
City	**Farm/House**		
Species	**House type**		
Category (meat, layer, breeder)	**Litter type**		
Breed	Stocking density		
Specific (sample level)			
Sample ID	Bird ID	Age (days)	Body weight (grams)
Collection date	Sex (male, female, mixed)	Diet/Feed	Antibiotic use
Health status	**Sample site (crop, gizzard, ileum, ceca, cloaca swab)**	**Sample type (content, mucosal scraping, excreta, ceca dropping)**	Sample mass

Fig. 2. Example of a metadata form for a poultry microbiome sample.

may be different across trials. Examples of trial level metadata include trial code, location (farm, city, state, country), house ID (within location), poultry species *(Gallus gallus domesticus, Meleagris gallopavo domesticus,* etc.), breed, chick source (hatchery), age of parent flock, and sample type (ceca content, fecal, cloaca swab, etc.), sample collection and storage (buffers, temperatures and time from collection to processing). There is also information related to the sample which is unique, this may include information such as bird ID, body weight (at sampling or at other time points), date and time the sample was taken, treatment the bird belongs to, diet being fed, sex, health status or other comments.

The distinction between sample identification and sample metadata, or trial level versus sample level metadata may vary depending on the trial goal and experimental design. For example, the metadata collected from a controlled research trial may be different from the metadata collected in a field study. Also, in a trial where different breeds are being compared breed is not part of trial level metadata but part of sample level metadata. Some metadata can be considered sensitive, thus if recorded, it may not be reported or published. Metadata collection, storage and use should comply with local and international regulations about personal and business private data protection laws. Private information may include customer names, location, personal data of the people involved or other information that if released may violate confidentiality agreements. Still, we suggest the use of a form that should collect minimal metadata for poultry microbiome studies. Fig. 2 presents an example of metadata form for poultry microbiome samples. We strongly recommend that this form be used whenever raw data is submitted to a public repository.

Sample collection and storage

These are a set of general guidelines that can be adapted to the experimental design, including the region from which the sample is collected, and where it is stored.

During experimental design

Sample collection is a direct manifestation of the hypothesis, and care should be taken during experimental design in deciding where, how, and why a sample is collected (Carr et al., 2019).

Invasive or non-invasive method of sample collection? Collecting samples from the region of interest remains the ideal practice for profiling microbial communities in the gastrointestinal tract of the bird. Nevertheless, non-invasive sample collection, e.g. excreta, cecal droppings or cloacal swabs, can be used but with the recognized disadvantage of not fully representing microbial communities in each GIT location.

From where should the sample be collected? When deciding on which gross GIT region of interest for sampling, the researcher should also determine if longitudinal differences (i.e. spatial variation along the length of any region from proximal to distal segments) exist in the region selected, for example proximal, mid, or distal portions of the ileum. Identify if any anatomical regions may present potentially confounding variables, such as the cecal tonsil, and decide if luminal content should be collected from, or away from, that section. If luminal content is desired, consider the method to collect. For example, the muscle in the gizzard can be more challenging for the collection of luminal content than simply severing a cecal pouch. If mucosal scrapings are desired, decide first if it is possible to differentiate from luminal content, and next what type of tool will be used to collect the scrapings. There are several methods available describing collection of mucosal scrapings (Awad et al., 2016; Campos et al., 2022; Gong et al., 2002, 2007; Hankel et al., 2022; Proszkowiec-Weglarz et al., 2022; Ruiz et al., 2015; van der Eijk et al., 2020).

Consumables, tools, and reagents. A positive control, as well as a negative control, should be incorporated into the sample collection procedure. Positive controls, e.g., mock bacterial community, are needed to ensure the quality of the data and to help assess the efficacy of sample extraction during downstream stages, while negative controls, e.g., an empty sample collection tube, are needed to identify any potential contamination. Sample collection tubes should be those that are designed and certified for DNA extraction (e.g. certified DNA and DNase free). Tubes should be labelled in a logical and clear fashion so that someone unfamiliar with the experimental design could understand what sample tubes contain. Tools should be sterilized before use in sample collection, to avoid possible sources of contamination. Still, tools should be cleaned and disinfected thoroughly to minimize the chances of cross-contamination. Molecular lab grade disposable materials are also preferred and recommended.

Although snap freezing is considered the gold standard for sample collection, field conditions may necessitate the use of a protective buffer to help prevent DNA degradation. If a buffer is used, it should be run alongside primary samples to test for any possible contamination.

During the experiment

Appropriate personal protective equipment (**PPE**) should be worn, depending on the experimental design and the biosafety level. Assuming correct PPE has been provided, sterile technique should be practiced during sample collection. Ideally, the same researcher should be tasked with the collection of the same sample type throughout the entire experiment to eliminate person-to-person variation. A dedicated set of tools should be utilized solely for opening the GIT location from which the sample is collected. If tools are reused between birds, tools should be first cleaned and then disinfected (e.g. 70% ethanol) before the tools are used on the next bird—this will help minimize any contamination among birds.

Immediately after euthanasia, the GIT should be removed, the gross section of interest visually identified, and the precise sampling location should be determined. If collecting luminal content, the gut section should be fully severed, the content gently squeezed directly into a sample collection tube followed by immediate snap freezing on dry ice or in liquid nitrogen. If collecting mucosal scrapings, one should first sever the gut region using a ball tip scissor and subsequently place the ball tip into the lumen and make a longitudinal cut along the mesenteric line so that the mucosa lies flat and is facing upwards. Gently, sterilized tweezers should be used to remove any luminal content, followed by using a scraping tool to make a single scrape unidirectional scrape (either left to right, or right-to-left) to collect mucosa (Campos et al., 2022; Proszkowiec-Weglarz et al., 2022; van der Eijk et al., 2020). Finally, the mucosal scraping should be snap frozen immediately. It is not recommended to flush the intestine to remove content before scraping as this could introduce contamination into the lumen.

After the experiment

All snap frozen samples should be stored in an ultra-low freezer (−80 °C) until ready to proceed with DNA isolation. Buffer stored samples should be stored according to buffer manufacturer instructions. Samples that are kept frozen in ultra-low freezers should not undergo freeze-thaw cycles. When thawing the sample for DNA extraction, the method used should be recorded.

A reporting checklist

Journals may have specific requirements for manuscripts containing microbiota data, for example Poultry Science has the following requirements. Authors should ensure that the Materials and Methods include all relevant sample collection and storage information, as illustrated in Fig. 3: a) the measured location of sample collection (e.g. approximately 3 cm distal from the Meckel's diverticulum); b) approximate sample mass (e.g. approximately 300 mg luminal content was collected); c) sample tube catalog number; d) age, sex, and breed of the

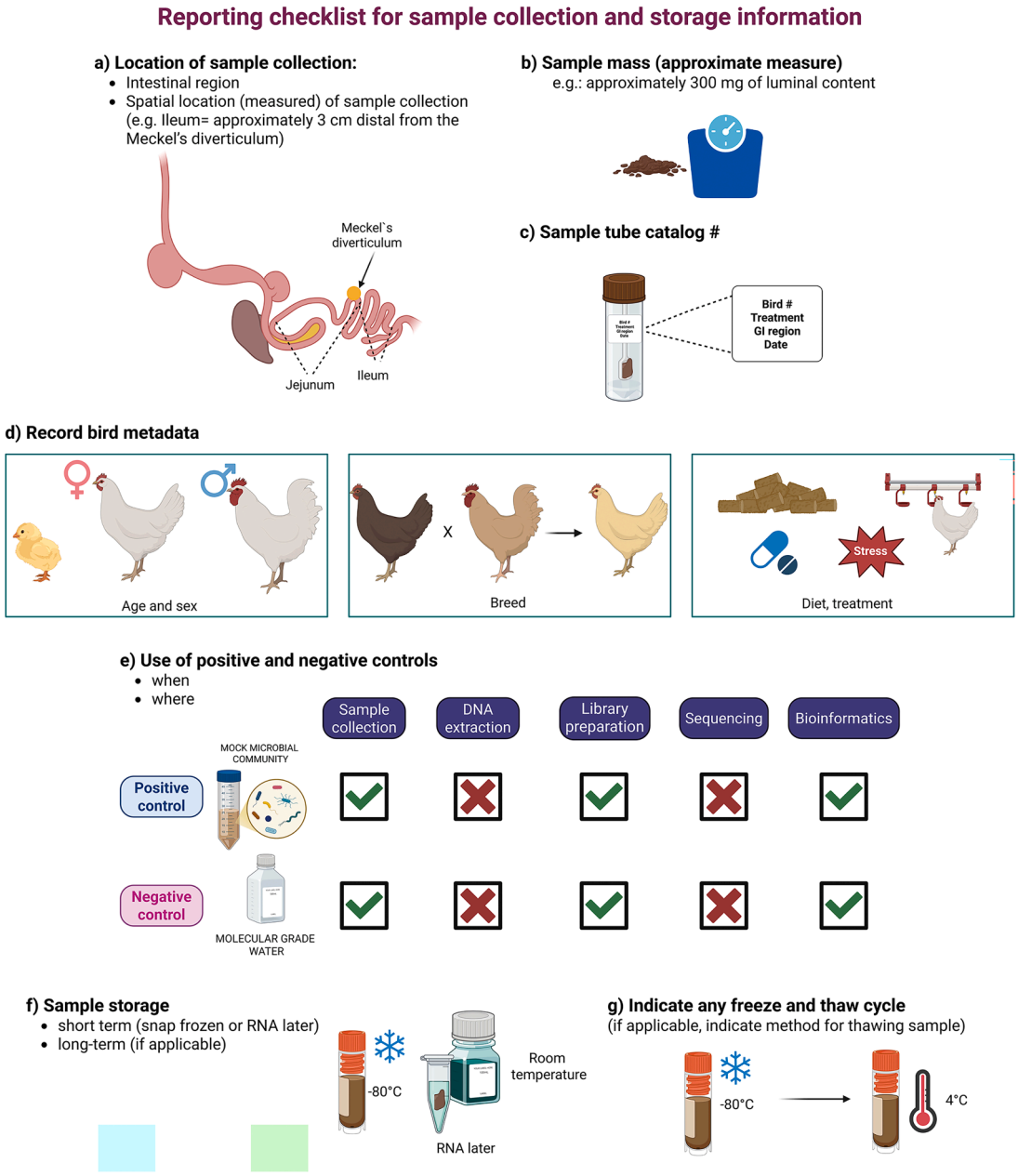

Fig. 3. Checklist for sample collection for microbiome analysis and storage information. Reporting checklist of steps that must be included in the Materials and Methods section of a manuscript submitted to *Poultry Science*, containing information on sample type, collection and storage. Created with BioRender.com.

bird; e) what, and where in the sample collection process positive and negative controls were used; f) storage method after collection; g) how were samples stored long-term, if applicable; h) indicate if the samples have undergone any freeze-thaw cycles; i) thaw method of frozen sample.

Sample size

The goal of microbiome studies is often to investigate, characterize, and understand the compositional and functional variability of microbial communities. This is commonly studied by hypothesis testing. Hypothesis testing relies on four key parameters: a) the effect size, which quantifies the outcome of interest, typically the difference between two groups; b) the sample size (n), referring to the number of observations collected; c) the statistical power ($1 - \beta$), representing the probability of correctly rejecting a false null hypothesis; and d) the significance level (α), which indicates the probability of incorrectly rejecting a true null hypothesis. Depending on the research question, the sample type and size will be different. Fig. 4 gives an overview of the many considerations to make when determining sample size in poultry microbiome research. First, scientists have to define the relative potential number of samples needed depending on the sample type of individual collected samples (experimental unit). Individual samples are a better choice for stages like the initial training of prediction models. Pooled samples, however, could be classified using an existing model with lower sample size.

Calculating sample size in microbiome studies is challenging due to the high dimensionality of sequencing data and the complex structure of microbial communities (Kers and Saccenti, 2021). Traditional power analysis methods used for univariate outcomes may not be directly applicable. Simulation methods provide a flexible framework to estimate power under realistic study conditions, especially when standard analytical formulas are insufficient (Arnold et al., 2011). There are tools that have been proposed to allow researchers to model realistic

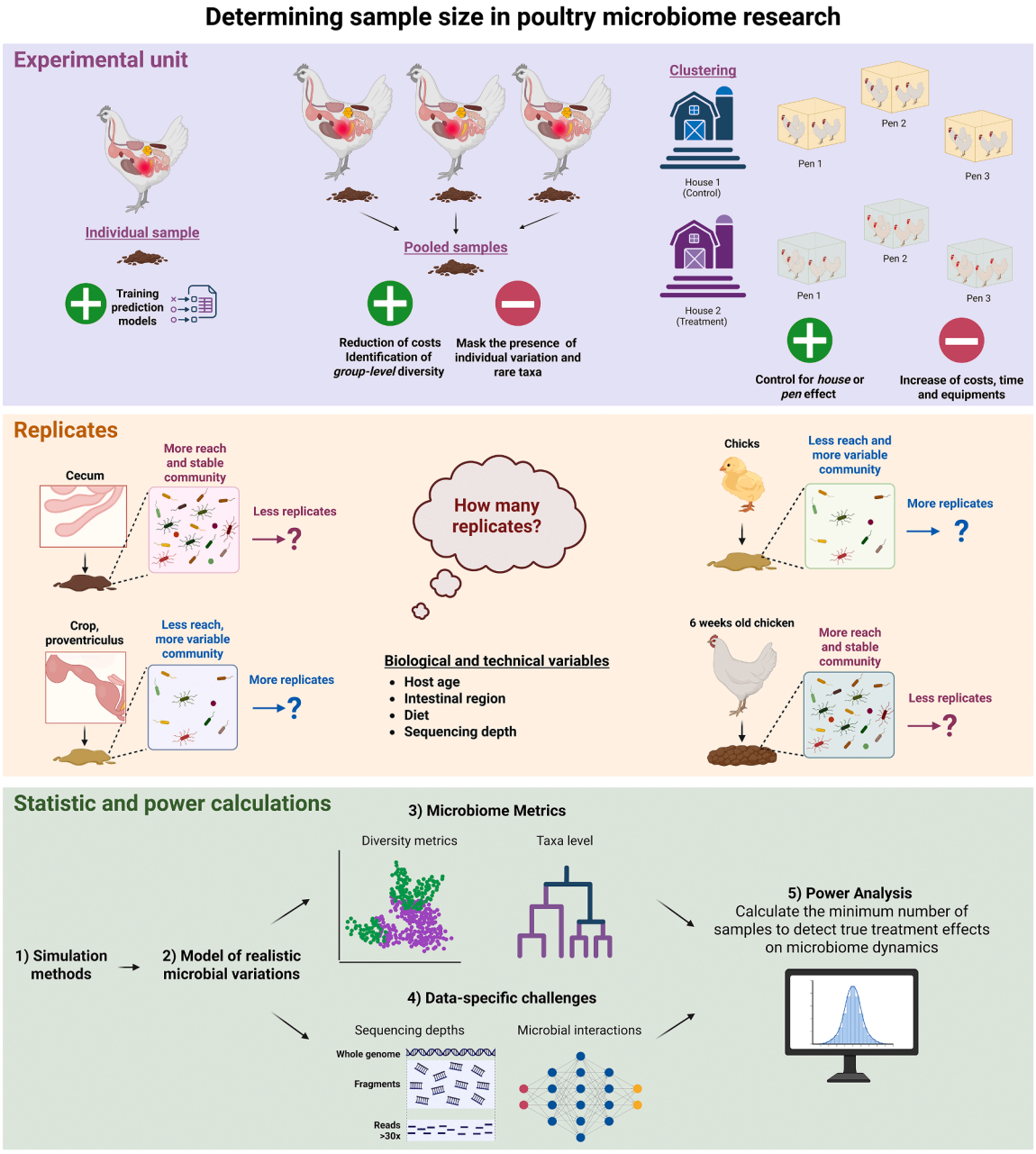

Fig. 4. Determining sample size in poultry microbiome research.
Schematic representation of key elements that contribute to the determination of the sample size in poultry microbiome research. In particular, biological variables can affect microbial composition and replicate variability whereas technical variables such as sequencing depth, databases and bioinformatic pipelines can influence data reproducibility and interpretation. Created with BioRender.com.

microbial variation and determine the probability of detecting an effect under different assumptions (Arnold et al., 2011; Chen, 2020; Ferdous et al., 2022; Kelly et al., 2015; La Rosa et al., 2012; Mattiello et al., 2016; Xia et al., 2018). In poultry research, additional considerations include clustering due to group housing or pen-based treatments. According to the Quadram Institute's guidelines, intra-class correlation (**ICC**) can substantially reduce the effective sample size if not adjusted for in the design (Savva, 2020). Power analysis must incorporate design effects for such clustered designs, and simulation becomes especially useful when modeling outcomes such as beta diversity or multivariate taxa shifts. Whether a study is exploratory, screening for patterns, or confirmatory, testing specific hypotheses also influences the type of power calculation needed and the level of acceptable uncertainty. Finally, employing a pilot study or using data from similar published research to estimate variance can improve sample size estimates. Careful planning of sample size not only strengthens the validity of conclusions but also enhances the reproducibility and translational value of poultry microbiome studies.

Determining the right sample size for microbiome studies requires balancing the logistical costs of sample collection, DNA extraction cost, and library preparation and sequencing cost with the need for statistical power to detect treatment effects. This balance is particularly nuanced in microbiome research, where biological variability, technical limitations, and experimental objectives interact. A critical first step is defining an independent sample, as failure to account for shared environmental exposures—such as animals housed in the same pen or repeated sampling from the same host—can result in pseudo replication and inflate significance (Reish et al., 2024). It is important to

acknowledge that sampling multiple animals within a pen is an acceptable practice and is not pseudo-replication (Colegrave and Ruxton, 2018; Davies and Gray, 2015; Schank and Koehnle, 2009). It is critical that the researchers design an experiment to account for within pen and potential between pen effects. Taking a lesson from the murine gut microbiota, intra versus inter cage effects were found to be dependent on cohort, cage density (e.g. how many mice per cage) as well as experimental hypothesis (Russell et al., 2022). Meaning, intra vs inter cage differences were not always observed. Hence in poultry, in addition to the experimental hypothesis, both the number of flocks (e.g. are multiple flocks being tested?), as well as pen density (e.g. how many birds per pen) should inform expectations of both within, and between, pen variation.

Once sample independence is confirmed, the next challenge is determining how many replicates are needed. While this might seem straightforward, factors such as host age, different parent flock and/or hatchery, and diet introduce considerable biological variability. In addition, sequencing platform choice, taxonomic database differences, and bioinformatic pipelines contribute to technical variability that can confound results (Lyte et al., 2025). As sequencing technologies and computational tools continue to improve, the resolution of microbial data and the reliability of sample size estimates are also expected to increase.

The microbiome metrics of interest, including alpha diversity, beta diversity, or taxonomic shifts can significantly affect the number of samples needed. For example, detecting changes in diversity indices typically requires different sampling depths and replication than identifying differentially abundant taxa (Debelius et al., 2016). Pooling strategies further complicate this decision. Pooling can reduce sequencing costs and capture a broader microbial snapshot from a given unit (e.g., a poultry pen), but this comes at the expense of losing individual-level resolution and the ability to detect low-abundance taxa (Weinroth et al., 2022a). Early attempts by Zhou et al. (2007), using denaturing gradient gel electrophoresis (**DGGE**), found pooling cecal samples from five broilers to be optimal. However, the increased resolution of 16S rRNA gene sequencing versus DGGE requires different pooling logic depending on the research aim.

Sample size needs also vary by anatomical site. The ceca, which typically harbor a taxonomically rich and relatively stable microbial community, may require fewer replicates than more variable sites such as the crop or ileum (Lyte et al., 2025; Weinroth et al., 2022a). In contrast, regions such as crop, proventriculus, duodenum, jejunum, ileum can better capture transient changes but often exhibit higher interindividual variability and, therefore, require more samples to account for potential noise. Casals-Pascual et al. (2020) showed that longitudinal sampling of fecal material can improve the reliability of diversity assessments, suggesting that temporal replication may reduce the need for larger cross-sectional sample sizes.

Sample size decisions in microbiome studies must consider independence, biological variability, sequencing depth, and analytical objectives. Fig. 4 summarizes key considerations such as pooling rationale, housing effects, and statistical approaches. Chen. (2020) noted that microbiome-specific data often requires larger sample sizes than other genomic studies due to greater variability and interdependence among microbial features. As sequencing platforms and bioinformatics methods evolve, sample size estimations will become more data-driven, enabling researchers to design studies that yield more accurate and biologically meaningful insights into microbial community dynamics. As a general guideline, we recommend collecting a minimum of 10-12 samples per treatment group, preferably from multiple pens or barns without pooling for 16S rRNA gene sequencing whenever feasible.

DNA isolation

For microbial DNA isolation from collected samples, commercial fecal or soil DNA isolation kits may be used such as Qiagen's PowerFecal Kits and Zymo Research's Quick-DNA Fecal/Soil Microbe Kits. These kits are designed for the rapid isolation of DNA (PCR inhibitor-free) from a variety of biological samples. To ensure consistent bacterial lysis among samples, it is important to use a similar amount of sample material across the treatments. Frozen samples should be thawed on ice, weighed, and adjusted to be within 20% variation and below a kit's maximum capacity. If all samples cannot be processed in a single run, we recommend processing samples in batches randomly across treatments, rather than one treatment at a time. Great care should be taken to minimize cross-contamination during sample processing. To minimize inter-individual variability, we recommend that all samples within a study be processed by a single experienced person whenever possible. Negative and positive controls should be included and processed for DNA extraction in the same manner as all other samples. The details are discussed later in this review.

Bead beating is crucial to mechanically disrupt difficult-to-lyse microorganisms before DNA extraction. The duration and intensity of bead beating need to be optimized for each instrument and sample type to minimize insufficient cell disruption or excessive DNA shearing. Some of the bead beating devices include MP Biomedical's FastPrep™, Benchmark Scientific's Beadblaster™, Qiagen's TissueLyser III, and Omni International's Bead Ruptor™. For samples with low microbial biomass, such as those from the respiratory, reproductive, and upper gastrointestinal tracts, it is beneficial to start with more material, pool if necessary, and elute DNA in a small volume of water. Zymo Research's Quick-DNA Fecal/Soil Microbe Microprep Kit is one such appropriate option.

For routine processing of large numbers of samples, consider using an automated DNA purification platform such as Qiagen's QIAcube™ or QIAsymphony™, Thermo Fisher Scientific's KingFisher systems, or Eppendorf's EpMotion™ liquid handling system. These automated systems are designed to streamline DNA extraction workflow, reduce hands-on time, and enhance consistency and reproducibility while reducing cross-contamination.

DNA quality and concentration

High-quality and accurately quantified DNA is critical for reproducible poultry microbiome studies, particularly in applications such as PCR amplification and 16S rRNA gene sequencing. DNA purity, quality and concentration influence downstream data quality, as contaminants such as proteins, phenols, and host DNA can interfere with amplification efficiency and microbiome profiling; it is worth noting that the presence of host DNA in intestinal luminal content is normal and should be expected due to epithelial cell turnover and other factors that have been recognized for decades (Hoskins, 1978). The removal of PCR inhibitors is critical for improving reproducibility and the detection of low-abundance taxa (Pankoke et al., 2021). Accurate measurement of DNA concentration and quality is fundamental to ensuring consistent results across studies and laboratories. Common methods for assessing DNA purity and concentration include spectrophotometry (e.g., NanoDrop) and fluorometry (e.g., Qubit), each with unique advantages and limitations. Spectrophotometric methods provide a quick estimation of DNA concentration through absorbance at 260 nm, while purity is typically assessed through A260/A280 and A260/A230 ratios (Huberman, 1995). For NanoDrop reading, A260/A280 ratios between 1.8-2.0 generally indicate DNA of acceptable purity, while A260/A230 ratios above 2.0 suggest minimal contamination with organic compounds and chaotropic salts used during extraction (Lim et al., 2020; Lucena-Aguilar et al., 2016). However, spectrophotometric methods alone are insufficient as they cannot discriminate among DNA, RNA, free nucleotides, and other contaminants, and may overestimate concentrations due to contaminants such as uric acid (Eriksson et al., 2017). Therefore, fluorometric quantification methods using dyes selective for double-stranded DNA, such as those employed in Qubit™ and PicoGreen™ assays, should be implemented to obtain more accurate

concentration measurements (Ban and Kim, 2024; Haque et al., 2003). Livestock microbiome research including pig and chicken samples has reinforced this conclusion, with Wegl et al. (2021) showing that combining both approaches minimizes errors and improves reproducibility across laboratories.

DNA integrity is often overlooked due to the time-consuming nature of gel electrophoresis and the cost of automated systems such as the Fragment Analyzer. However, for critical applications, especially when working with challenging samples such as those from poultry litter or intestinal contents with high PCR inhibitor content, gel electrophoresis should be performed to assess DNA integrity and fragment size distribution (Stellwagen, 2009). Additionally, quantitative PCR (**qPCR**) targeting the 16S rRNA gene can provide valuable information on the amplifiability of the extracted DNA and help identify samples with high levels of PCR inhibitors or host DNA contamination (Jian et al., 2020; Opel et al., 2010).

For poultry microbiome studies, we recommend implementing standardized thresholds for DNA quality and concentration: a minimum final DNA concentration requirements should be optimized based on the specific sequencing chemistry and targeted genomic regions being analyzed, with typical ranges of 5 to 10 ng/μL for library preparation and at least 50 to 100 ng of total DNA available for multiple analyses serving as general guidelines; purity measurements showing A260/A280 ratios between 1.8-2.0 and A260/A230 ratios above 1.8; integrity characterized by minimal fragmentation as assessed via gel electrophoresis or automated systems (e.g., Agilent Bioanalyzer or TapeStation) with a predominant band above 10 kb for optimal results; and demonstrated amplifiability through successful 16S rRNA gene amplification confirmed by qPCR, with Ct values comparable to those obtained with control DNA of known quality.

To enhance transparency and reproducibility, researchers should report detailed information on the methods used for DNA quality and concentration assessment (including instrument models and settings), the actual measured values for concentration (as ng/μL), A260/A280 and A260/A230 ratios, and any other quality metrics employed, the criteria used for determining the suitability of DNA samples for downstream analyses, and the percentage of samples that failed quality control along with the reasons for exclusion (Gerasimidis et al., 2016; Panek et al., 2018). Recent poultry microbiome studies have demonstrated that adherence to strict DNA quality standards significantly reduces technical variability and improves reproducibility across different laboratories (Feye et al., 2020b; Fidler et al., 2020).

Primer selection

Amplicon-based 16S rRNA gene sequencing remains one of the most widely used approaches for characterizing microbial communities, including those in the poultry GIT. This method relies on PCR amplification of hypervariable regions (V1–V9) within the highly conserved 16S rRNA gene. Primers are designed to anneal to conserved flanking regions, enabling targeted amplification of the variable areas where taxonomic information is concentrated. Choosing which hypervariable region to target and which primer pair to use has significant implications for taxonomic resolution, detection sensitivity, and analytical bias (Pollock et al., 2018).

Importance of primer selection

Primer selection is a critical and non-trivial step in 16S rRNA gene amplicon study design. Differences in primer binding efficiency across microbial taxa can lead to skewed community profiles by disproportionately amplifying certain groups while excluding others. These biases are particularly consequential in poultry microbiome studies, where Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria dominate the microbial community (Aruwa et al., 2021). For example, some primer sets underrepresent Clostridiales or Campylobacteraceae, which play essential roles in poultry health and food safety (Feye et al., 2020b; Lyte et al., 2025). Additionally, suboptimal primer selection may result in excessive co-amplification of host DNA or increased formation of chimeric sequences, thereby reducing read quality and usable data yield (Olson et al., 2025; Souza et al., 2023).

The performance of primer sets varies markedly depending on the taxa of interest and the sample type (Johnson et al., 2019). In their comparative benchmarking study, Johnson et al. (2019) demonstrated that the 515F–806R (V4) primer pair yielded the highest species richness and diversity estimates across diverse environmental samples, offering broad taxonomic coverage that included key poultry-associated phyla. The 341F–805R (V3–V4) primers provide particularly good resolution for Proteobacteria and Bacteroidetes, making it a solid choice for GIT microbiome studies. In contrast, 787F–1073R (V5–V6) offers enhanced detection of Actinobacteria and Verrucomicrobiota, however, at the cost of reduced overall diversity estimates. The 799F–1193R (V5–V7) set is well-suited for plant-associated microbiomes due to its ability to reduce chloroplast DNA amplification, but it has limited utility in animal microbiome research. Weinroth et al. (2022a) further emphasized that primer selection must be tailored to the specific research objectives and the microbial communities of interest. The authors suggest using mock communities and standardized controls to assess primer performance and identify amplification bias. Additionally, the authors encourage researchers to consider region-specific community differences when selecting primers, especially within different sections of the poultry GIT.

Primer sets used in poultry microbiome research

Poultry microbiome studies have historically used a range of primer pairs targeting different hypervariable regions, including V1–V3, V3–V4, and V4, as well as full-length 16S rRNA gene regions (Oakley et al., 2014). Although the selection has often been study-specific, the field has begun moving toward greater standardization to improve reproducibility and cross-study comparability. Lyte et al. (2025) emphasized that a lack of consistency in primer selection has contributed to discrepancies in microbial profiles across studies. Weinroth et al. (2022a) also highlighted that primer choice should reflect the anatomical specificity of the microbial niche under investigation. For instance, cecal, ileal, and crop communities differ in composition, necessitating deliberate primer targeting. The authors recommend that studies document primer sequences, amplification parameters, and DNA extraction methods to ensure reproducibility and comparability. Table 1 provides a comprehensive summary of primer sets—including target regions, performance characteristics, poultry-specific applications, and supporting references—which integrates benchmarking data with use cases relevant to poultry microbiome studies.

Recommendations for standardization

The poultry GIT microbiome is primarily composed of obligate anaerobes from the families Lactobacillaceae, Clostridiaceae, and Ruminococcaceae, with additional contributions from Enterobacteriaceae and Bacteroidaceae (Feye et al., 2020a; Lyte et al., 2025). Given the predominance of these taxa and the technical considerations of sequencing workflows, it is recommended to standardize poultry-focused 16S rRNA gene amplicon studies around the V3–V4 region. This recommendation aligns with prior benchmarking studies and the consensus emerging in the field (Pollock et al., 2018; Yeh et al., 2018). The V3–V4 region offers several key advantages. For example, an intermediate amplicon length (~460 bp) that enhances resolution without exceeding read limits for common short-read platforms. Other benefits include improved performance across dominant poultry-associated phyla and compatibility with major databases such as SILVA and Greengenes, which support robust taxonomic assignment. Moreover, Weinroth et al. (2022a) stressed the importance of

Table 1
16S rRNA gene primer sets – performance and poultry applications.

Primer Pair	Target Region	Amplicon Length (bp)	Strengths	Limitations	Application Context	References
27F-338R	V1-V2	250	High genus/species-level resolution for some taxa; compatible with Illumina	Primer bias against some groups; limited universality compared to V3–V4	Useful for targeted studies needing high taxonomic resolution; limited but growing use in poultry microbiomes	(Abellan-Schneyder et al., 2021; Borda-Molina et al., 2019; Pineda-Quiroga et al., 2019)
27F–534R	V1–V3	500–550	Captures early gut colonizers; used in early studies	Overestimates diversity; biased against Firmicutes	Occasionally used in poultry; historical relevance	(Li et al., 2018; Oakley et al., 2014; Zhao et al., 2013)
341F-534R	V3	~190	Good resolution of *Firmicutes* and *Bacteroidetes*; short-read compatible	Very short fragment may reduce phylogenetic resolution	Occasionally used in high-throughput settings where read length is highly constrained	(Cao et al., 2018; Klindworth et al., 2013; Zhao et al., 2013)
515F-806R	V4	250-300	High species richness; broad coverage including *Firmicutes* and *Bacteroidetes*	May underrepresent *Actinobacteria*; short read may miss strain-level detail	General use, comparative studies, and some poultry microbiomes	(Pollock et al., 2018; Xu et al., 2018; Yeh et al., 2018; Zhang et al., 2018; Zhao et al., 2013)
341F–805R	V3–V4	460	Good resolution of Proteobacteria and Bacteroidetes; Illumina compatible	Slightly lower diversity estimates than V4	Recommended standard for poultry microbiome studies	(Feye et al., 2020a, 2020b; Metzler-Zebeli et al., 2019a, 2019b; Pollock et al., 2018; Weinroth et al., 2022b)
515F-926R	V4-V5	~400	Improved resolution across Firmicutes, Proteobacteria, and Bacteroidetes; moderate read length for MiSeq	May underrepresent Verrucomicrobia; intermediate amplicon size may limit full overlap in paired-end reads	Used in gut and environmental poultry microbiomes; balances read length with taxonomic resolution	(Parada et al., 2016; Wang et al., 2018)
515R-1061R	V4-V6	~550	Covers three hypervariable regions, improving phylogenetic resolution; good for long-read overlap	May have reduced coverage for specific taxa; longer amplicon less compatible with short-read platforms like MiSeq	Suitable for platforms supporting longer reads (e.g., PacBio, Nanopore); used in diverse and phylogenetic studies	(Wang and Qian, 2009) (Tremblay et al., 2015; Zhao et al., 2013)
787F–1073R	V5–V6	280–290	Better detection of Actinobacteria and Verrucomicrobia	Lower overall diversity estimates; possible bias	Less common in poultry; used for niche taxa studies	(Kers et al., 2019; van der Eijk et al., 2020; Yeh et al., 2018)
799F–1193R	V5–V7	390	Minimizes chloroplast DNA amplification	Limited bacterial group coverage; low diversity estimates	Ideal for plant-associated samples	(Yeh et al., 2018)
27F-1492R	V1-V9	~1500	Maximal taxonomic resolution; spans all hypervariable regions; ideal for phylogenetics and strain-level ID	Requires high-quality DNA; longer and more expensive sequencing (e.g., PacBio, Nanopore platforms)	Long-read sequencing for high-resolution profiling; emerging in poultry and microbiome reference studies	(Benítez-Páez et al., 2016; Johnson et al., 2019)
27F–1492R	Full-length	~1500	Maximal taxonomic resolution; ideal for phylogeny	High-quality DNA is needed; a costlier long-read platform	Long-read (PacBio, Nanopore); emerging in poultry	Benítez-Páez et al. (2016)
8F–1391R	Near full-length	~1380	Broad-range detection; full-gene coverage	Potential primer mismatches in certain taxa	Long-read or clone-based profiling; moderate poultry use	Frank et al. (2008)

transparent reporting standards—including primer sets, target region, sequencing chemistry, and data processing steps—to facilitate methodological comparisons and meta-analyses.

Future directions: transition to long-read 16S rRNA gene sequencing

With more accurate and accessible long-read sequencing technologies, full-length 16S rRNA gene sequencing is poised to become a new standard for microbial community analysis (Olson et al., 2025). Platforms such as PacBio and Oxford Nanopore now allow for near-complete coverage of the 1.5 kb 16S rRNA gene, enabling taxonomic resolution at the species or even strain level (Benítez-Páez et al., 2016). These long-read approaches also improve phylogenetic reconstructions and reduce biases in targeting specific hypervariable regions. However, widespread adoption of long-read 16S rRNA gene workflows remains constrained by cost, error rates (particularly in Nanopore sequencing), and limited database support compared to short-read approaches (Frank et al., 2008; Lyte et al., 2025; Olson et al., 2025). As curated full-length reference datasets improve and error-correction algorithms mature, full-length 16S rRNA gene sequencing will likely become more viable for routine poultry microbiome studies. Weinroth et al. (2022a) recommended integrating long-read data with short-read platforms to enhance microbial resolution and support taxonomic continuity. The authors also called for community-driven efforts to develop validated protocols and standardized bioinformatics workflows to facilitate widespread adoption.

Positive and negative controls

Positive and negative controls in microbiota research refer to defined standards containing either whole cell or DNA mixtures. Positive controls can be used to assess efficacy at each stage of a microbiota study ranging from DNA isolation kit performance to bioinformatic tool bias. Negative controls are utilized to assess contamination, and like positive controls can be integrated into any stage of the experimental design. Positive and negative controls play a key role in reproducibility, accuracy, and other important aspects of microbiota data analysis and interpretation (Hornung et al., 2019). It is, therefore, our express recommendation that positive and negative controls are incorporated in the experimental design of all poultry microbiota studies (Lyte et al., 2025).

The 'where' and 'how' regarding implementation of positive and negative controls

Locations to include positive and negative controls within a poultry

study workflow have been previously discussed (Lyte et al., 2025). In brief, a positive and/or negative control can be used during a) sample collection, b) sample storage, c) DNA isolation, d) library preparation, e) sequencing, and f) data analysis and processing. Although the use of a separate control for each experimental stage can provide insight into how that particular step may perform or be a source of contamination, it may not always be feasible to have multiple positive and negative controls in a single study. As such, it is recommended that at minimum, a single positive control and single negative control be included at the first stage of the experiment, namely sample collection, and then processed alongside actual samples through the entire project pipeline. This methodology has the advantage of providing a holistic perspective on how each step (e.g. from sample collection to storage to DNA isolation, and so on) impacts the final results.

The 'what' regarding choice of positive and negative controls

The selection of a positive control must be determined by two factors. The first is at what stage, and for what purpose, in the experiment is the positive control being used. The second factor is if the mock community suitably represents the microbial communities in the collected samples. The choice of a whole cell mixture is suitable up to and including DNA isolation. A DNA mix standard can be used starting at library preparation, and a mock community dataset can be utilized at a bioinformatic stage. Nevertheless, it must be made clear that a mock whole cell mix can be carried through the entire experimental pipeline. A whole cell mix can be used during extraction of DNA, that then can be carried through to library preparation and sequencing, and a dataset be generated for bioinformatic processing. Hence, it is up to the researcher where the positive control(s) may be used most effectively to assess for performance or bias in any single or multiple experimental stage(s).

The choice(s) of a negative control is more numerous, as contamination can come from the environment as well as from consumables and kits. Hence, the researcher must determine which step of the experimental pipeline carries the greatest concern for contamination. Like positive controls, the researcher has two complementary routes to integrate a negative control: The first is to use a control during sample collection and carry it through the entire downstream process up to and including bioinformatic processing. The second is to use a negative control at any single experimental stage. The use of molecular grade water, for example, in an empty sample tube can be used during sample collection, then included alongside primary samples in all processing steps including being run through the same DNA extraction kit, undergo identical library preparation, and be part of the same sequencing run.

Using positive and negative control data

There is no consensus on how positive control data should be used to inform data interpretation. A theoretical relative percentage of a mock whole cell or DNA standard mixture is not expected to be exactly reproduced in actual results. Rather it should be expected that, at a minimum, the actual results capture all taxa included in the standard. It is up to the researcher to determine an acceptable discrepancy between theoretical and the actual relative percentages of each microbial genus. It is best practice to report the theoretical and actual results of positive control (Shetty et al., 2023).

Negative control data can identify contamination that can be removed at the bioinformatic stage. Nevertheless, the researcher should proceed with caution when removing any sequence, having a clear justification for the removal of reads. Negative controls through the subtraction of contaminating reads have been instrumental in identifying the role of consumables and reagents in creating the appearance of low biomass microbiomes, such as blood, brain, and placental microbiotas (Kennedy et al., 2023). It is best practice to report how the negative control data was used, the reads that the negative control produced, as well as what reads have been removed and for what reason (s).

A checklist for reporting positive and negative controls

The researcher should ensure proper reporting of the use of positive and negative controls in submitted manuscripts, relevant metadata, and deposited raw datasets. A simple checklist should include: a) What positive and negative controls were used, along with catalog #'s; b) How, and at what experimental stage(s), were the positive and negative controls included; c) Brief reasoning as to why the controls were used at what stage(s); d) Present results of the positive control against theoretical composition in the manuscript; e) Detail how negative control data were handled, and if and why reads were determined to be contaminants; f) Include raw positive and negative controls' data in the project deposited in a data repository.

Library preparation and quality

DNA samples should be quantified and diluted to a desired concentration.Then DNA should be used for the amplification of the V3–V4 hyper variable region of bacterial 16S rRNA gene by using the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′). After amplification, amplicons should be checked in 2% agarose gels to verify the fragments' size, the expected size is ~ 550 bp. Amplicons should then be purified using a commercial DNA purification kit such as AMPure XP beads (Beckman Coulter) as per manufacturer's recommendations, and dual indices and Illumina adapters from the Nextera UD index set (Illumina, San Diego, CA, USA) should be added for index PCR. Amplified libraries should then be purified, using AMPure XP beads, and quantified in triplicates using a Qubit fluorometer with a dsDNA HS Assay kit (Invitrogen, Carlsbad, CA, USA). Libraries can be validated by running a 1 μL aliquot on a Bioanalyzer (Agilent Technologies, Palo A, California) or using QIAxcel DNA Hi Resolution cartridge, QIAxcel ScreenGel 1.6.0 software, and QIAxcel Advanced System (Qiagen) per manufacturing instructions to verify the size of the fragment ~ 630 bp. After quantification, libraries should be diluted to 4 nM, to generate equivalent number of raw reads, and aliquots of 5 μL of each diluted library should be used to create a pool of libraries, the number of libraries to be pooled depends on the coverage needs. Lastly, pooled libraries should be diluted to a final concentration of 6 pM, and spiked with 5% PhiX control (Illumina, San Diego, CA, USA) before sequencing using a MiSeq Reagent kit v3 (600 cycle) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

Sequencing platform

Detailed discussions regarding the comparative strengths and limitations of different sequencing technologies have been presented in our previous review (Lyte et al., 2025). Here, we specifically provide protocol recommendations for the widely utilized short-read Illumina and long-read Oxford Nanopore sequencing platforms.

Illumina sequencing technologies, particularly the MiSeq, HiSeq, and NovaSeq systems, have dominated poultry microbiome research over the past decade due to their high accuracy (>99.9%) (Logsdon et al., 2020) and substantial throughput capabilities. We recommend paired-end sequencing with a read length of 250 or 300 bp (PE250 or PE300) for 16S rRNA gene amplicon sequencing These paired-end reads provide adequate overlap to assemble sequences of the frequently targeted V3-V4 regions. It is noted that V3-V4 sequencing may have limitations on the resolution of taxonomic assignments, particularly among closely related bacterial species prevalent in poultry microbiomes.

Long-read sequencing using PacBio or Oxford Nanopore Technologies (**ONT**) has emerged as a promising approach for achieving superior taxonomic resolution in poultry microbiome research. Both, PacBio and Nanopore sequencing can generate long reads spanning the entire 16S rRNA gene (approximately 1,500 bp) and even larger genomic

fragments, significantly enhancing the capacity for precise taxonomic identification and facilitating the discovery of novel microbial taxa (Cuscó et al., 2018; Szoboszlay et al., 2023). Recent advancements in PacBio and Nanopore sequencing chemistries have further reduced the cost and error rates (Wan et al., 2022), making both technologies increasingly practical for routine applications in poultry microbiome studies.

The sequencing platform should be selected early in study planning as it influences numerous upstream and downstream protocol decisions, and significantly influences the accuracy, depth, and comprehensiveness of microbial community profiling in poultry microbiome research. Several critical considerations should be considered when selecting a sequencing platform for poultry microbiome research. Research objectives significantly influence the sequencing platform decision. Illumina platforms are generally suitable for exploratory analyses and routine monitoring, whereas PacBio and Nanopore platforms are better suited for detailed taxonomic characterization and novel species discovery (Dommann et al., 2024; Egeter et al., 2022; Szoboszlay et al., 2023). Additionally, the selection of sequencing platform also affects sample processing, such as ONT require high molecular weight DNA with minimal shearing, and Illumina platforms are more tolerant of fragmented DNA but require stricter purity standards.

Sequencing depth is a crucial consideration in microbiome research, as it significantly influences the accuracy of microbial diversity assessments. Previous studies suggest that sequencing depths of approximately 10,000 to 20,000 high-quality sequences per sample are generally sufficient for reliable characterization of bacterial diversity (Lundin et al., 2012; Schloss, 2024). While increased sequencing depths (20,000 to 50,000 reads per sample) enhance the detection of rare microbial taxa, they may offer limited additional value for overall diversity evaluations (Cameron et al., 2021; Caporaso et al., 2011). Conversely, sequencing depths below 5,000 reads per sample are likely to underestimate true microbial diversity (Lundin et al., 2012). Therefore, we recommend that researchers use rarefaction curves and Good's coverage estimates to ensure they are sequencing to a depth appropriate to capture the majority of diversity in their sampling matrix, as sequencing depth requirements vary considerably depending on the bacterial diversity within the sampled environment (Schloss, 2024). Table 2 summarizes the output specifications of commonly used sequencing platforms to assist researchers in selecting appropriate technology and determining optimal sample pooling strategies based on study requirements.

To improve comparability across poultry microbiome studies, detailed methodological reporting is strongly recommended. Researchers should include comprehensive information regarding sequencing platforms, chemistry versions, read configurations (single or paired-end), and achieved sequencing depths per sample (Abundo et al., 2021; Klair et al., 2023). Reporting platform-specific quality control metrics, such as the percentage of reads achieving Q30 quality for Illumina and mean quality scores for ONT, is also beneficial.

Emerging sequencing platforms such as Illumina NextSeq 2000, NovaSeq X, and ONT PromethION continue to evolve, providing improved combinations of accuracy, read length, and throughput. Poultry microbiome researchers should stay abreast of these advancements to leverage new technologies effectively as they become available.

Bioinformatic analysis

Among the most widely used open-source bioinformatic pipelines for the analysis and interpretation of 16S rRNA gene amplicon sequencing data are QIIME 2 (Bolyen et al., 2019), MOTHUR (Schloss et al., 2009), and UPARSE (Edgar, 2013). The pipeline of QIIME 2 is often used due to its comprehensiveness, integrating numerous plugins and hundreds of bioinformatic packages, along with detailed tutorials and documentation for data analysis. QIIME 2 begins with the demultiplexing, trimming, and quality filtration of single or paired-end raw FASTQ sequence data to remove barcodes, adaptors, and low-quality sequences. The sequences are then dereplicated to create a non-redundant set of sequences. Denoising is further performed using either the Deblur (Amir et al., 2017) or DADA2 plugin (Callahan et al., 2016) to generate amplicon sequence variants (**ASVs**) and a feature table that summarizes ASV frequency across samples. These ASVs can be used to generate a rarefaction curve to assess whether the sequencing depth was sufficient to capture the overall diversity of the microbial community in each sample. Rare ASVs, such as singletons, those present in $< 5\%$ of samples, or those with an average relative abundance of less than 0.01%, could be contamination and not a biological signal. Therefore, those ASVs are often discarded. We recommend indicating average sequencing reads $\pm$ SD per sample and the number of ASVs obtained before and after rare ASV removal. The retained ASVs are taxonomically classified using reference databases such as SILVA (Quast et al., 2013), RDP (Wang and Cole, 2024), and Greengenes2 (McDonald et al., 2024). Ultimately, a new feature table is generated with the relative abundances of feature sequences in each sample, assigned at different taxonomic levels (usually phylum, family, genus, and species). The relative abundance results can be presented in the form of tables, stacked bar graphs, or pie charts. It is worth highlighting that the output of sequencing, without accompanying flow cytometry or other quantitative techniques, is relative abundance (Lin and Peddada, 2020a; Props et al., 2017).

Given that sequencing often produces varying read depths across samples, data normalization is critical to ensure unbiased comparisons among samples. Common normalization methods include rarefying and cumulative sum scaling (**CSS**) (Prodan et al., 2020) . Rarefying is achieved by subsampling to a smaller, predetermined count, typically the fewest sequence read count among the samples, while CSS adjusts counts based on the cumulative sum (Lin and Peddada, 2020b). Data normalization is typically performed before α- and β-diversity analysis and differential abundance testing. Normalization strategies may need to account for differences between high-biomass samples (e.g. cecal

Table 2
Comparison of sequencing platform specifications.

	Short read (Illumina)			Long read (Nanopore)		
Platforms	MiSeq	MiSeq i100 Plus	NovaSeq 6000	PromethION	GridION	MinION
Flowcell type	MiSeq V3	50 M	SP	PromethION	MinION/Flongle	MinION/Flongle
Flowcell number per run	1	1	1-2	1-48	1-5	1
Read length (bp)	2 × 300	2 × 300	2 × 250 [2]	1500 [3]	1500	1500
Max data output per flowcell	15 Gb	30 Gb	400 Gb	100 Gb	35 Gb [4]	2.6 Gb [5]
Max reads per flowcell	25 M [1]	100 M	800 M	66 M	23 M	1.7 M

Note:.
[1] For MiSeq Reagent Kit v3 only. M: million reads.
[2] NovaSeq 6000 read length and output varies by chemistry version; only SP flowcell produces 2 × 250 bp reads.
[3] For Nanopore platforms, 1500 bp is a typical average read length for 16S rRNA gene amplicon sequencing studies, but read length can be much longer (>100 kb).
[4] Standard Flongle flowcell.
[5] Standard MinION flowcell.

content) and lower-biomass samples (e.g. ileal content), where low read counts may influence normalization choices and downstream diversity estimates.

The α-diversity (within-sample diversity) of a microbiota sample is measured by the richness and evenness of individual bacterial taxa, as well as their phylogenetic relationships. Each of these aspects can be measured using multiple indices. For instance, bacterial richness can be indicated by observed ASVs, evenness by Pielou's or Simpson's Evenness index, and phylogenetic relationships by Faith's phylogenetic diversity. The Shannon Index, Simpson's Diversity Index, and Inverse Simpson's Index consider both richness and evenness to measure the overall α-diversity of a sample. It is noted that, due to the removal of singletons, Chao1 and ACE are not suitable for estimating richness of ASV datasets (Deng et al., 2024)

The β-diversity (between-sample diversity) can be assessed using the Jaccard Index, Bray-Curtis Dissimilarity Index, Aitchison distance, and Unweighted and Weighted UniFrac Distances. The Jaccard Index measures the similarity between two samples based on the presence or absence of species, while the Bray-Curtis Index accounts for both the presence/absence and abundance of species. The Aitchison distance is the Euclidean distance for center log-ratio (**CLR**) transformed abundances, aiming to avoid the compositionality bias. Unweighted UniFrac measures the distance between two samples based on the evolutionary relationships of the species present or absent, whereas Weighted UniFrac also considers the relative abundance of species (Lozupone and Knight, 2005).

We recommend showing the impact of the treatments on both α- and β-diversities of the samples. Generally, one metric, but not multiple metrics, is sufficient to indicate each of the richness, evenness, and α-diversity. To visualize β-diversity dissimilarities among multiple samples, principal coordinates analysis (**PCoA**) or non-metric multidimensional scaling (**NMDS**) is used to demonstrate either, but not both sets, of Jaccard/Bray-Curtis indices or Unweighted/Weighted UniFrac among samples. Hierarchical clustering and heatmaps are commonly employed to display the patterns and relationships among differentially abundant bacterial taxa. Other advanced bioinformatic analyses such as PICRUSt2-based functional prediction of the microbiome (Douglas et al., 2020), co-occurrence network analysis, and machine learning can be applied, dependent upon specific research needs. These methods are beyond the scope of this current recommendation, and readers should refer to other publications (Regueira-Iglesias et al., 2023).

Operational taxonomic units (OTUs) vs. amplicon sequence variants (ASVs)

Historically, operational taxonomic units (**OTUs**), defined by clustering similar sequences at approximately 97% similarity, served as the primary approach for microbiome analyses (Knight et al., 2018; Pollock et al., 2018). OTUs emerged primarily to mitigate sequencing errors by grouping reads and averaging out minor discrepancies (Schloss et al., 2009). However, the OTU method exhibits significant limitations, such as potential loss of biological resolution due to clustering (Callahan et al., 2017; Edgar, 2018). Such limitations mean OTUs cannot reliably distinguish closely related microbial species and yield results specific to particular datasets or clustering algorithms, complicating cross-study comparisons.

Recent methodological advances have introduced ASVs, which represent exact sequence variants corrected for sequencing errors using computational algorithms like DADA2, Deblur, and UNOISE (Amir et al., 2017; Callahan et al., 2016; Edgar, 2016). These algorithms employ statistical models to differentiate true biological variation from sequencing errors, achieving single-nucleotide resolution without arbitrary similarity thresholds (Callahan et al., 2016). A comparative study of these three algorithms concluded that DADA2 gave the best biological resolution at the cost of producing higher number of spurious ASVs while USEARCH-UNOISE3 showed the best overall combination of sensitivity and specificity (Prodan et al., 2020).

Consequently, ASVs represent actual biological sequences observed within samples, improving taxonomic resolution, reproducibility, and comparability between studies (Callahan et al., 2017). However, their use has sparked debate; for example, (Schloss, 2021) demonstrated that ASVs may artificially split sequences from the same genome into separate clusters due to intragenomic variation in 16S rRNA gene copies. Despite this limitation, ASVs remain valuable for detecting subtle ecological differences and resolving closely related taxa in complex microbial communities (Callahan et al., 2017; Knight et al., 2018).

Comparative evaluations of OTUs and ASVs indicate clear advantages of ASVs (Table 3) (Prodan et al., 2020). Firstly, ASVs substantially enhance taxonomic and ecological resolution, capturing fine-scale microbial variations that OTU clustering typically obscures (Callahan et al., 2017). This resolution is particularly important in poultry microbiome studies, where even single-nucleotide differences can signify biologically significant distinctions among taxa like *Campylobacter* or *Clostridium* species (Oakley and Kogut, 2016). Moreover, ASVs ensure reproducibility across datasets, allowing direct comparisons and integration of findings across different studies (Callahan et al., 2017). In contrast, OTUs, being study-specific, hinder cross-study meta-analysis and cumulative knowledge advancement (Callahan et al., 2016).

While ASVs have some limitations, such as potentially inflating diversity due to recognizing multiple closely related variants, these issues are manageable through appropriate bioinformatic analyses, such as subsequent aggregation at a suitable taxonomic level if desired (Callahan et al., 2017). Practically, ASV methods are computationally efficient and scalable, enabling robust analyses even with large sequencing datasets (Callahan et al., 2016).

The implications for various poultry-related sample types, including cecal, ileal, fecal, and litter samples, reinforce the preference for ASVs. ASVs provide consistent, high-resolution microbiome profiling across these diverse samples, accurately distinguishing subtle differences and preventing misclassification of environmental contaminants versus true gut microbes (Fasolo et al., 2024; Oakley and Kogut, 2016; Stanley et al., 2015). The high resolution offered by ASVs is particularly valuable for studies assessing gut-environment microbial interactions and ensuring accurate representation of microbial complexity across different poultry-associated sample types (Fasolo et al., 2024).

Regarding sequencing methodologies, Illumina-based sequencing targeting regions such as V3–V4 remains common, and ASV methods are particularly well-suited to these short-read data (Callahan et al., 2016).

Table 3
Comparison of operational taxonomic units (OTUs) vs amplicon sequence variants (ASVs) in 16S rRNA gene analysis.

Feature	OTUs (97% Clustering)	ASVs (Denoising)
Definition	Clusters of sequences at ≥97% similarity	Exact biological sequences after error correction
Resolution	Genus/approximate species level	Single nucleotide differences
Reproducibility across studies	Low – OTUs are study-specific	High – identical ASVs represent the same organism
Handling of sequencing errors	Averaged out in clusters	Modeled and largely removed
Diversity estimation	May underestimate richness (Fasolo et al., 2024)	Often improves richness and resolution (Callahan et al., 2017; Fasolo et al., 2024)
Computational efficiency	Less efficient for large datasets	Scales linearly with sequence depth
Cross-study comparison	Difficult, require re-clustering	Direct comparison of sequence identifiers
Application in low biomass samples	May introduce spurious variants	Better filtration of errors and contaminants (Callahan et al., 2016)
Suitability for poultry gut samples	Less accurate for complex communities	Optimal for all sample types

However, newer long-read sequencing technologies (e.g., PacBio, Oxford Nanopore) offer opportunities to generate full-length 16S rRNA gene sequences (Callahan et al., 2019). These platforms further improve species-level classification accuracy, and applying ASV approaches tailored to their unique error profiles enhances microbiome characterization (Callahan et al., 2019). The development of full-length ASV analyses represents a promising direction for achieving even higher resolution and accuracy in microbiome studies (Callahan et al., 2019).

Considering these methodological advancements and empirical findings, we strongly recommend adopting ASV-based analytical approaches as the standard for poultry 16S rRNA gene microbiome research. ASVs provide accurate, reproducible, and ecologically meaningful microbial community characterizations, enabling detailed comparative analyses. Future studies should implement rigorous ASV pipelines, thoroughly report exact sequences, and integrate ASVs into diversity and functional analyses. This approach maximizes data utility, reproducibility, and cross-study comparability, fundamentally improving our understanding of poultry microbiome ecology and facilitating comprehensive, integrated microbiome research across the community.

Database selection

The choice of database for 16S rRNA gene taxonomic classification directly affects the accuracy, resolution, and reproducibility of outputs. Several databases are available, each with different coverage, update frequency, and compatibility with analysis pipelines. In poultry gut microbiota studies where community composition includes a wide range of taxa, database choice can influence the detection and classification of both common and less abundant microbes. One important consideration in database selection is how each database incorporates recent taxonomic nomenclature updates. Since 2021, NCBI Taxonomy has undergone substantial revisions, including the addition of numerous new prokaryotic phyla and systematic renaming to follow standardized nomenclature rules (Oren et al., 2021; Oren and Garrity, 2021; Schoch et al., 2020). These changes particularly affect bacterial classification and can impact how poultry-associated taxa are named and reported. Users should be aware that different databases may adopt these changes at different rates, potentially leading to inconsistencies when comparing results across studies or databases.

SILVA

SILVA (Quast et al., 2013) is one of the most widely used and regularly updated databases. It contains curated ribosomal RNA sequences from all domains of life and provides good genus- and species-level resolution. SILVA includes many sequences relevant to avian gut microbiota, making it suitable for poultry studies. Its large size can increase computational requirements, and its taxonomy may differ from older literature, so users should be aware of naming changes when comparing with previous studies.

Ribosomal database project

The Ribosomal Database Project **(RDP)** (Cole et al., 2014; Wang et al., 2007) is also commonly used and provides a well-curated set of sequences along with a naïve Bayesian classifier. RDP is relatively small in size, which makes it faster to use, and its taxonomy assignments are consistent. However, it is updated less often than SILVA, which can limit coverage of recently described poultry-associated taxa.

Greengenes

Greengenes (DeSantis et al., 2006) was widely used in the past but has not been updated since 2013. This makes it less suitable for current research, given the substantial taxonomic changes and species discoveries in recent years.

Greengenes2

Greengenes2 (McDonald et al., 2024) is a newer release that improves on the original Greengenes by using an updated phylogenetic approach. However, its effectiveness for poultry microbiome analysis has not been extensively validated.

Other databases, such as EzBioCloud (Yoon et al., 2017), contain well-curated type strain sequences and can improve species-level resolution in some cases. EzBioCloud is partly subscription-based and less frequently used in standard open-source workflows, which may limit its use in some settings. Databases such as GTDB (Parks et al., 2018) focus on whole-genome taxonomy rather than amplicon sequences, making them better suited for shotgun metagenomics than for 16S analysis.

For poultry microbiome studies, SILVA is recommended as the primary database due to its broad coverage, frequent updates, and good representation of avian-associated taxa. RDP can be used when faster processing or comparisons with earlier RDP-based studies are needed. The original Greengenes should be avoided, though Greengenes2 can be tested in parallel for comparison. Whatever database is chosen, the version, release date, and any changes made during analysis should be clearly reported to allow reproducibility.

Statistical analysis

Microbiome data derived from 16S rRNA gene sequencing are inherently compositional and interdependent, characterized by frequent deviation from normality, and a high prevalence of zero counts and outliers. Consequently, statistical analyses typically employ nonparametric methods unless the assumptions of normality and homogeneity of variance are satisfied. For comparisons of α-diversity indices, the Kruskal-Wallis test is appropriate for multi-group comparisons, while the Wilcoxon rank-sum test is suitable for pairwise analyses. For β-diversity analyses, permutational multivariate analysis of variance (**PERMANOVA**) (Anderson, 2001) is a widely employed as a robust nonparametric approach, implemented via the adonis function in the vegan package (Oksanen et al., 2019).

Dozens of differential abundance analysis (**DAA**) methods have been developed for microbiome data. Several recent comparative evaluations have demonstrated that no single method consistently outperforms others across all scenarios (Cappellato et al., 2022; Nearing et al., 2022; Yang and Chen, 2022). Therefore, it is advisable to apply multiple DAA methods to derive consensus findings or to select methods tailored to the study design (e.g., cross-sectional vs. longitudinal) and data characteristics (e.g., sparsity, compositionality). In general, parametric or nonparametric statistical methods that do not control false discovery rate (**FDR**) are inappropriate for identifying differentially abundant taxa. Among the currently available methods, ALDEx2, MaAsLin2, and ANCOM-BC perform reasonably well, while DESeq2 and edgeR—originally developed for RNA-sequencing data analysis— are generally discouraged for microbiome analysis due to their lack of compositional awareness and inadequate handling of zero inflation (Weiss et al., 2017).

Recently developed DAA techniques such as ANCOM-BC2 (Lin and Peddada, 2024) and MaAsLin3 (Nickols et al., 2024) offer improved performance by adjusting for compositional bias and effectively controlling FDR, and are therefore strongly recommended. On the other hand, Linear Discriminant Analysis Effect Size (**LEfSe**), although widely used (Segata et al., 2011), is not recommended for multiple-group comparisons due to its lack of FDR correction, sensitivity to data sparsity, and failure to account for compositionality, which may lead to false positives, particularly among low-abundance taxa. If LEfSe is used for two-group comparison, we recommend $P < 0.05$ and a $\log_{10}$ LDA score of ≥ 2.0 or 3.0 as the threshold for statistical significance.

For correlation analyses between microbial relative abundances and

host phenotypic traits, methods specifically designed for microbiome data are preferred over conventional Pearson or Spearman correlations, which do not account for compositionality and are sensitive to sparsity and zero inflation. Recommended tools include SparCC (Watts et al., 2019), SPIEC-EASI (Kurtz et al., 2015), and SPRING (Yoon et al., 2019). Both *P*-values and correlation coefficients (e.g., *R*-values) should be reported to convey the strength and significance of associations.

Data deposition

Deposition of raw data into public repositories has become a common prerequisite to manuscript acceptance in peer-reviewed journals. Public repositories provide researchers with reliable long-term data storage and enable easy data sharing within the scientific community. Meta-analyses using data from public repositories have widened our understanding of the presence of common intestinal microbial taxa (Cardenas et al., 2021), the role of microbiota in various intestinal infections (Pietruska et al., 2023), as well as the effect of sampling method on microbiome composition (Zhou et al., 2023). For data to be most useful long-term, it is recommended that researchers follow a few specific guidelines.

Scientists should begin by choosing a free public repository to allow data to be most accessible to the larger scientific community. The authors recommend using one of the three established International Nucleotide Sequence Database Collaboration (**INSDC**) (Cochrane et al., 2016) databases. These include the National Library of Medicine Sequence Read Archive (**NCBI-SRA**) (Katz et al., 2022), the European Nucleotide Archive (**ENA**) (Yuan et al., 2024), and the DNA Databank of Japan Sequence Read Archive (**DDBJ-DRA**) (Mashima et al., 2017). INSDC databases are not only publicly available but are also automatically synced increasing global connectivity and providing important data back-ups. Deposited data should be raw, which is defined here as the data that is supplied directly from the sequencer or the sequencing company. If the data has been pre-processed in any way (i.e. demultiplexing, removal of barcodes and primers, etc.), it should be mentioned in the project summary portion of the submission. Deposition of raw data will enable it to be more effectively reanalyzed with future analysis techniques allowing for maximal impact and longevity of the data. It is recommended that individual sequencing files be deposited for each sample along with sequencing files for negative controls and any internal or positive controls used.

Along with raw sequencing data, the metadata form recommended in this manuscript should also be included in the data deposition. At publication, the name of the chosen public genomic database and accession number or link associated with the data should be reported in the manuscript.

Conclusions

Approaching the microbiota in both experimental design and analysis can be a daunting task. The researcher is presented with an array of design considerations, reagents, and methodologies at each experimental stage from initial tissue sampling through data analysis and choice of raw data repository. Variation in results among studies can generate the appearance of both inconsistency and irreproducibility in poultry microbiota research. However, it is fundamentally important to appreciate that inconsistency among studies does not necessarily mean results are irreproducible, but rather that the results are a product of the methodology used. Further, although there are many methodological choices for the poultry microbiota researcher, this does not necessarily mean one methodology is superior to another. Simply put, there is no singular methodology that is best suited for generating reproducible, robust, and consistent microbiota results. Rather, the experimental design, selected reagents, bioinformatic choices, and other aspects must be informed by a best practices framework that should be universally implemented in poultry microbiota research. And, those methodological choices should be reported in sufficient detail to enable reproducibility. Moreover, whatever methods are used, they should be communicated in detail so that if differences occur within the literature, the methodology is sufficiently documented to determine if the methodology is a factor. It is the express hope of the present manuscript that a best practices approach, including the use of positive and negative controls, improved sharing of raw primary and meta-datasets, in addition to other aspects, can quickly and compellingly enhance the pillars of robustness, reproducibility, and consistency in poultry microbiota research.

CRediT authorship contribution statement

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Disclosures

Conflicts of interest statement: The authors declare no conflicts of interest.

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