

COMPARED APPROACHES FOR CHARACTERIZING RUMEN MICROBIOTA: CULTURE-BASED, MOLECULAR, AND QUANTITATIVE PERSPECTIVES

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ABSTRACT

A comprehensive understanding of rumen microbial communities is critical for improving feed efficiency, reducing methane emissions, and enhancing sustainable livestock production. Recent advances in molecular technologies have revolutionized the analysis of rumen microbiota, encompassing culture-based, molecular, and quantitative approaches. This review aimed to compare these approaches to provide a more comprehensive, accurate, and reliable understanding of the rumen microbiota. Conventional culture-based techniques enable the direct isolation and physiological characterization of microorganisms, but they can identify only a limited proportion of microbes that grow under laboratory conditions. Molecular approaches enable comprehensive microbial profiling, although they are often semi-quantitative. Among these, 16S rRNA gene-based methods provide detailed insights into the composition and diversity of microbial communities, including uncultured bacteria and archaea. Nevertheless, these methods are susceptible to amplification bias and generally lack absolute quantification. Quantitative polymerase chain reaction (qPCR), in contrast, offers sensitive and accurate absolute quantification of specific microbial targets but is limited to known microorganisms and requires appropriate standards. Metagenomics approaches provide the most comprehensive analysis by revealing both the taxonomic composition and potential genetic roles of microbial communities. However, metagenomics remains expensive, requires complex bioinformatics analysis, and typically does not provide absolute quantification without integration with other techniques. Therefore, contemporary rumen microbiome research commonly employs a combination of these techniques to obtain a more comprehensive understanding of the rumen ecosystem.

Keywords: culture-based techniques, metagenomic, rumen microbial communities, revolutionized molecular approaches

INTRODUCTION

The rumen microbiota plays a fundamental role in feed digestion and metabolism in ruminants, directly influencing nutrient utilization, animal productivity, health, and environmental sustainability. This highly complex microbial ecosystem consists of bacteria,

archaea, protozoa, fungi, and viruses that interact synergistically to degrade fibrous plant materials and convert them into volatile fatty acids, microbial protein, and other metabolites essential for the host animal (Mayulu and Christiyanto, 2025). Given its central importance, a

comprehensive understanding of rumen microbial communities is critical for improving feed efficiency (Keum et al., 2024), reducing methane emissions (Lai et al., 2025), and promoting sustainable livestock production (Novikova et al., 2026).

Traditionally, studies of rumen microbiota relied heavily on culture-based methods (Denman et al., 2018), which enabled the isolation and physiological characterization of specific microbial species. These approaches have provided valuable insights into microbial metabolism, enzymatic activity, and functional roles. However, because a large proportion of rumen microorganisms are difficult or impossible to culture under standard laboratory conditions, culture-dependent techniques provide only a partial view of the rumen ecosystem. It has been estimated that only about 20% of rumen microorganisms can be cultured using conventional techniques (Matthews et al., 2019). This limitation arises because many rumen microbes are obligate anaerobes that require highly specialized cultivation conditions, while microbial interactions within the rumen are highly complex (Gilbert et al., 2020). Furthermore, the growth and survival of rumen microbiota are strongly influenced by microbial syntrophy—the phenomenon in which microbes exchange metabolic products such as hydrogen. Consequently, many microbial species depend on metabolites produced by neighboring organisms (cross-feeding), making them difficult to cultivate in isolation (Pitta & Narayan, 2022).

The development of molecular technologies has revolutionized rumen microbiology by allowing culture-independent characterization of microbial diversity and community structure (O'Hara et al., 2023; Prakash et al., 2023; Autenrieth et al., 2025; Sibilio et al., 2025). Techniques such as polymerase chain

reaction (PCR), quantitative PCR (qPCR), fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and next-generation sequencing (NGS) have greatly expanded current understanding of microbial composition and community dynamics. More recently, advanced omics approaches, including metagenomics, metatranscriptomics, and metabolomics, have provided deeper insights into microbial functions, metabolic pathways, and microbial interactions within the rumen environment.

In addition to microbial identification, quantitative approaches are increasingly recognized as essential for interpreting the biological significance of microbial community changes. Relative abundance data alone may not accurately reflect microbial activity or actual population shifts, whereas absolute quantification methods can provide a more accurate assessment of microbial load, ecological interactions, and microbial responses to dietary or environmental interventions (Wang et al., 2021; Gao et al., 2025).

Despite substantial technological progress, each methodological approach possesses inherent strengths and limitations. Culture-based techniques provide functional validation but are limited in scope; molecular methods offer broad taxonomic coverage but may not accurately indicate microbial viability or activity; and quantitative approaches improve ecological interpretation but require rigorous methodological standardization. Therefore, comparing culture-based, molecular, and quantitative approaches is essential for obtaining a more comprehensive, accurate, and reliable understanding of rumen microbiota, ultimately supporting the development of strategies to improve animal productivity, animal health, and environmental sustainability.

SEARCH STRATEGY

This review included studies on molecular analyses of the rumen microbiota, encompassing culture-based, molecular, and quantitative approaches from 2009 to 2026. Several scientific

databases, including PubMed, ScienceDirect, Scopus, Web of Science, and Google Scholar, were searched for terms relating to the issue covered in this review.

CHARACTERIZING MICROBIOTA APPROACH

a. Conventional culture-based methods

Conventional culture-based techniques are used to isolate and cultivate microorganisms under controlled laboratory conditions. These methods enable the biochemical, physiological, and morphological characterization of rumen microorganisms. Although culture-based approaches have contributed substantially to understanding the functional properties of rumen microbes, they do not fully represent the diversity of the rumen microbiota because the majority of rumen microbes are difficult or impossible to culture under standard laboratory conditions (Denman et al., 2018). Nevertheless, these techniques remain crucial for investigating microbial metabolism, metabolic pathways, and

generating reference genome maps from isolated strains.

Conventional culturing methods are based on the ability of microorganisms to proliferate on artificial media. Rumen fluid is generally collected aseptically and processed under strict anaerobic conditions using an anaerobic chamber or roll-tube techniques. Depending on the analytical goals, serial dilutions are prepared and inoculated into selective media designed for specific microbial groups, such as cellulolytic, amylolytic, or proteolytic microorganisms. Following incubation, microbial populations are quantified as colony-forming units (CFU), followed by preservation and characterization of the isolates (Figure 1) (Aldridge et al. 2016, Denman et al. 2018, and Prakash et al. 2023).

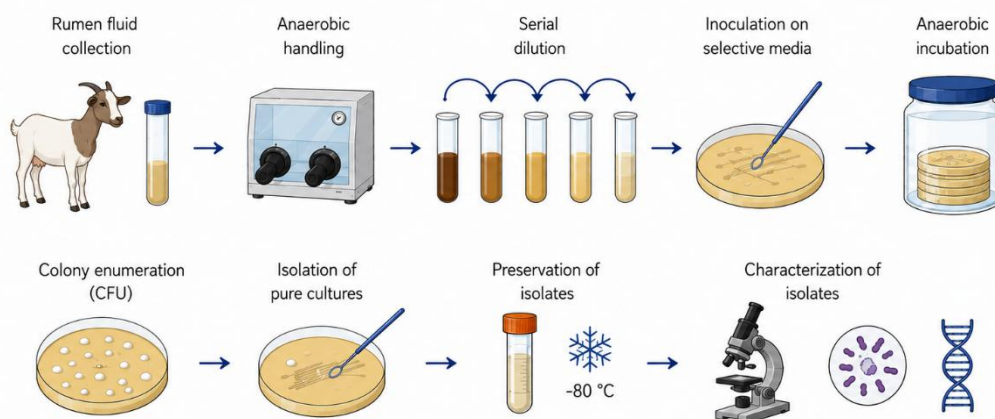


Figure 1. Workflow of conventional culture-based techniques for the isolation, enumeration, and characterization of rumen microorganisms (Aldridge et al. 2016, Denman et al. 2018, and Prakash et al. 2023).

One example of rumen bacterial isolation is *Ruminococcus* spp., including

R. albus and *R. flavefaciens*, which can be isolated from ruminal contents under strict

anaerobic conditions. The isolates can be screened for cellulolytic activity using carboxymethyl cellulose (CMC) Congo red agar. The formation of a clear zone indicates β -D-glucan degradation and cellulolytic activity (Figure 2) (Alam et al., 2024).

From an experimental perspective, culture-based methods are highly valuable for isolating microbial strains, directly examining metabolic activities, and studying microbial physiology. However,

these approaches are subject to substantial selection bias because less than 10% of rumen microbes are considered culturable under conventional laboratory conditions. Consequently, culture-based methods primarily represent the "culturable fraction" of the rumen ecosystem rather than the entire microbiome, making them less suitable for comprehensive community-level characterization (Creevey et al., 2014; Seshadri et al., 2018).

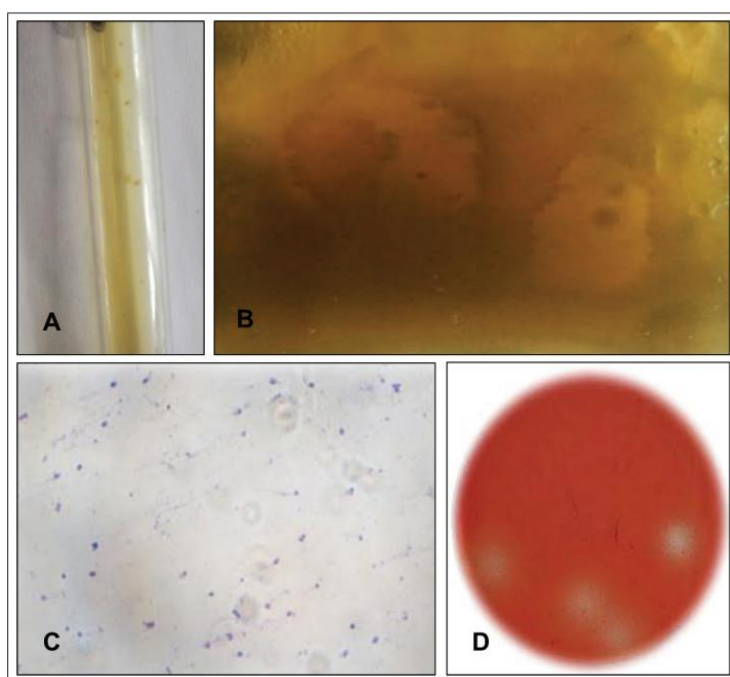


Figure 2. Isolation and characterization of cellulolytic *Ruminococcus* spp. from the rumen of Sahiwal cattle: (A) yellowish colonies of isolated *Ruminococcus* spp.; (B) clear zone around colonies indicating cellulolytic activity; (C) cellular morphology observed at 100 $\times$ magnification; and (D) clear zone following Congo red staining, indicating β -D-glucan degradation (Alam et al. 2024).

b. Molecular-based approaches

16S rRNA gene sequencing

The 16S rRNA gene-based approach has become a standard method in contemporary microbial ecology for identifying bacterial and archaeal communities. The 16S rRNA gene contains conserved regions that serve as primer-binding sites and intervening hypervariable regions that provide taxonomic information, enabling the characterization of microbial community composition using universal or broadly

conserved primers (Klindworth et al., 2013). In this technique, DNA is extracted from rumen samples and amplified using universal primers targeting conserved regions flanking hypervariable regions of the 16S rRNA gene, most commonly the V3-V4 regions (McGovern et al., 2018). The amplified products are subsequently analyzed using next-generation sequencing (NGS) platforms, such as Illumina sequencing (Figure 3). Following sequencing, bioinformatics pipelines involving operational taxonomic unit

(OTU) or amplicon sequence variant (ASV) clustering and taxonomic assignment are used to characterize

microbial community structure (Caporaso et al., 2010; Callahan et al., 2016).

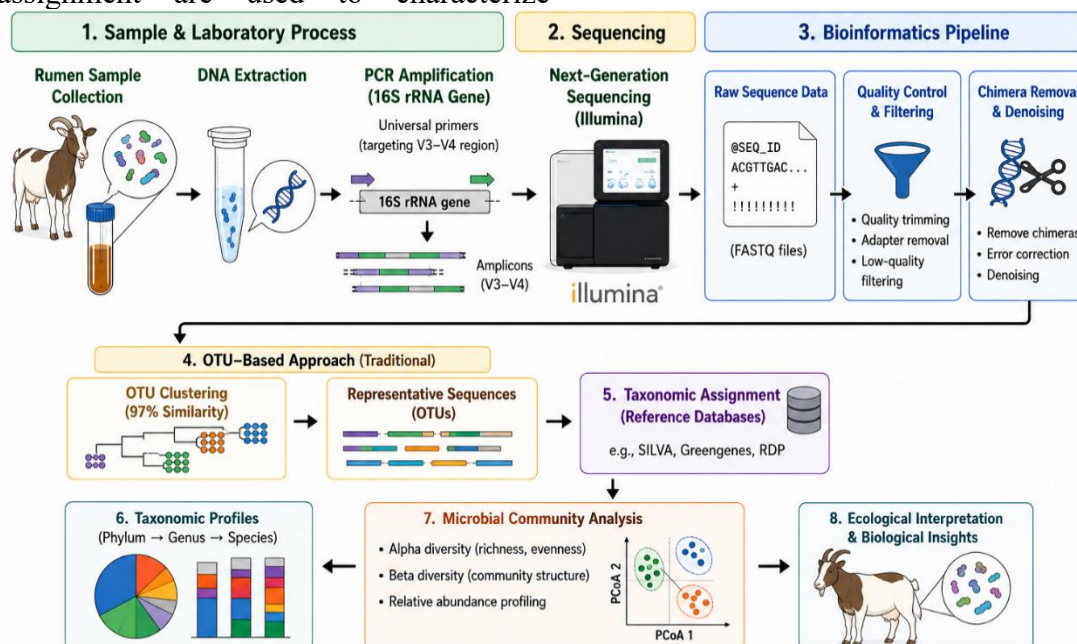


Figure 3 Workflow of 16S rRNA gene-based analysis for rumen microbial community profiling (Caporaso et al. 2010; Callahan et al. 2016; McGovern et al. 2018).

This approach enables comprehensive profiling of the rumen microbiome, including the large proportion of unculturable microorganisms that cannot be detected using conventional culture-based methods. As a result, 16S rRNA sequencing has substantially expanded current understanding of rumen microbial diversity and community structure. However, the method generally provides relative rather than absolute quantification of microbial abundance. Furthermore, interpretation of the data may be influenced by several factors, including PCR amplification bias, primer specificity, amplification efficiency, and variation in 16S rRNA gene copy number among microbial species. Consequently, 16S rRNA sequencing is frequently combined with complementary quantitative approaches to improve the accuracy and biological interpretation of microbial community analyses (O'Hara et al., 2023).

Shotgun Metagenomic Sequencing

Shotgun metagenomic sequencing represents one of the most comprehensive approaches currently available for microbiome analysis. Unlike targeted 16S rRNA sequencing, metagenomics involves random sequencing of all genomic DNA present within a rumen sample without prior target amplification (Prakash et al., 2023). This approach enables simultaneous characterization of taxonomic composition and functional potential, including genes, enzymes, and metabolic pathways associated with fiber degradation, methanogenesis, and other rumen metabolic processes.

Metagenomic analyses also facilitate the discovery of new genes and provide insights into microbial evolution and horizontal gene transfer within the rumen ecosystem. By sequencing entire microbial genomes, shotgun metagenomics contributes to a deeper understanding of rumen microbial ecology and functional interactions (Xu et al., 2026). However, this approach remains associated with several

limitations, including high sequencing costs, substantial computational requirements, complex bioinformatics analyses involving genome assembly, binning, and annotation, and challenges related to host DNA contamination. Similar to 16S rRNA sequencing, metagenomic data are generally semi-quantitative unless combined with additional normalization or quantitative methods (Singh et al., 2014).

Metatranscriptomics (RNA-seq)

Metatranscriptomics involves the analysis of total RNA to identify genes that are actively expressed within microbial communities. While metagenomics reveals the functional potential of a microbiome, metatranscriptomics provides insights into real-time microbial activity and functional responses under specific environmental or dietary conditions (Dai et al., 2025). This approach is particularly valuable for understanding dynamic microbial processes and metabolic regulation within the rumen ecosystem.

c. Quantitative methods

Quantitative PCR (qPCR) and Digital PCR (dPCR)

Quantitative PCR (qPCR) provides a sensitive and target-specific method for the absolute quantification of microbial populations in the rumen. In this approach, DNA is first extracted from rumen samples and amplified using specific primers targeting microbial marker genes, such as bacterial 16S rRNA, archaeal marker genes, protozoal 18S rRNA, and fungal marker regions. During PCR amplification, the accumulation of amplified products is monitored in real time through fluorescence detection, allowing the abundance of target microbial genes to be quantified. For absolute quantification, standard curves are generated using serial dilutions of DNA standards containing known concentrations or known numbers of target gene copies,

and the resulting quantification cycle values are used to estimate the number of target gene copies in rumen samples. Recent studies have applied qPCR to quantify major ruminal microbial groups, including total bacteria, archaea, protozoa, and fungi, with microbial abundance expressed as gene copies relative to the amount of extracted DNA or rumen sample (Dunière et al., 2025). Similarly, qPCR using group-specific primers has been employed to quantify total bacteria, archaea, and protozoa in rumen fluid, demonstrating its utility for monitoring changes in ruminal microbial populations (Hao et al., 2025). Target-specific qPCR has also been developed to detect and quantify specific ruminal bacteria, such as *Fusobacterium necrophorum* and *Fusobacterium varium*, using species-specific genetic targets (Deters et al., 2024).

Quantitative PCR is particularly useful for testing specific biological hypotheses, such as evaluating the effects of dietary interventions on methanogen populations or monitoring shifts in selected microbial groups. However, qPCR is limited to predefined targets and therefore does not provide comprehensive community-level characterization. In addition, the conversion of gene copy number to actual cell abundance should be interpreted cautiously because microbial species differ in genome size and gene copy number (da Silva-Marques et al., 2018; Singh et al., 2014).

Digital PCR (dPCR) is a targeted molecular technique that enables the absolute quantification of specific nucleic acid sequences without requiring a standard curve. Unlike qPCR, which quantifies target DNA based on fluorescence accumulation during amplification and typically relies on a standard curve, dPCR partitions the reaction mixture into thousands of individual microreactions before amplification. Each partition is subsequently classified as positive or negative based on the presence or absence

of a fluorescence signal, and the proportion of positive and negative partitions is analyzed using Poisson statistics to determine the absolute concentration of the target nucleic acid (Kuypers & Jerome, 2017; Huggett & Whale, 2018). This partition-based approach allows dPCR to provide direct absolute quantification without external calibration standards and reduces the influence of variations in amplification efficiency on the final quantification.

The qPCR and dPCR are targeted methods for measuring specific genes or

microbial populations. The comparative characteristics of these approaches are presented in Figure 4. These techniques overcome the limitations of relative abundance in sequencing-based methods by offering absolute quantification (Autenrieth et al., 2025). Digital PCR generally provides improved precision and accuracy compared with qPCR and can offer higher analytical sensitivity, particularly for low-abundance targets and samples affected by PCR inhibitors (Kuypers & Jerome, 2017; Quan et al., 2018; Sidstedt et al., 2020).

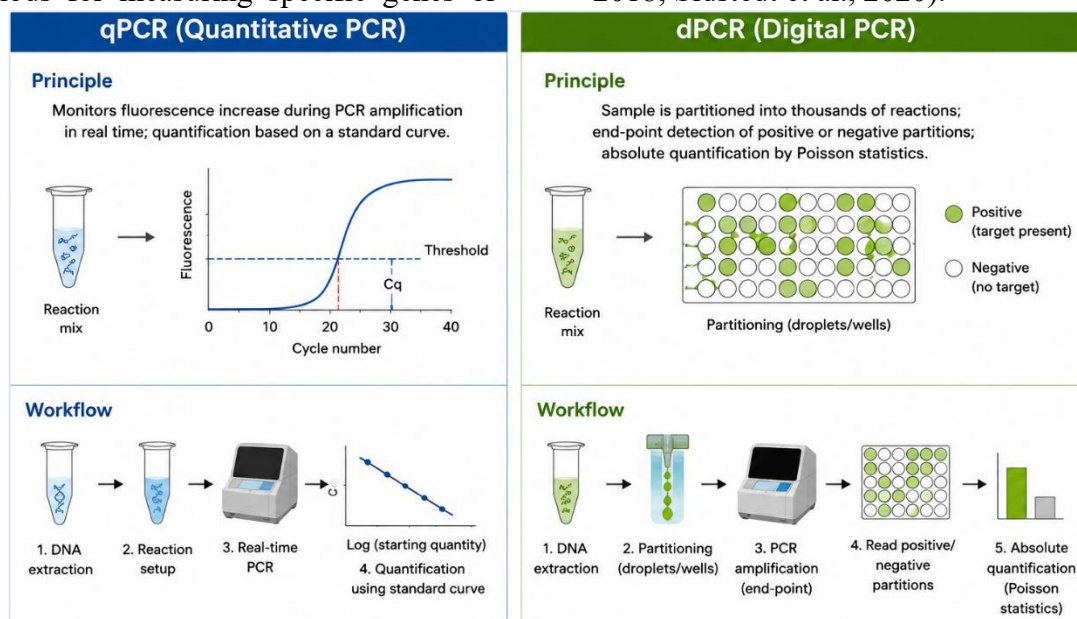


Figure 4. Schematic comparison of the principles and workflows of quantitative PCR (qPCR) and digital PCR (dPCR). qPCR quantifies target DNA by monitoring fluorescence in real time and generally relies on a standard curve, whereas dPCR partitions the sample into multiple individual reactions and determines absolute target concentration from endpoint-positive partitions using Poisson statistics (Kuypers and Jerome 2017; dMIQE Group 2020).

Fluorescence In Situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) utilizes fluorescently labelled probes targeting specific rRNA sequences to visualize microorganisms directly within their natural environment. This technique enables the identification, localization, and spatial analysis of microbial populations within the rumen ecosystem. However, FISH has several limitations, including relatively low throughput and the requirement for prior knowledge of target

sequences for probe design (Autenrieth et al., 2025).

Flow cytometry

Flow cytometry enables rapid quantification and characterization of microbial cells based on their physical and fluorescence properties. This technique supports high-throughput single-cell analysis and can be combined with FISH to improve taxonomic specificity and functional characterization of microbial populations (Tin et al., 2025). Recent

technological advances, including spectral flow cytometry, have enhanced the ability to directly detect and quantify rumen methanogens and other microbial groups within highly complex rumen samples.

d. Integration of multi-omics approaches

Modern rumen microbiome studies increasingly integrate multiple omics approaches to overcome the limitations associated with individual methodologies. For example, combining metagenomics and metatranscriptomics enables differentiation between the functional potential and the active metabolic functions of microbial communities. Furthermore, integrating these approaches with quantitative techniques, such as qPCR or dPCR, improves the accuracy of microbial abundance estimation and ecological interpretation (Sibilio et al., 2025).

Advantages and limitations of each method
Each method used in rumen microbiome research possesses distinct advantages and limitations, and these approaches are often complementary. Conventional culture-based techniques are highly valuable for the direct isolation and physiological characterization of microorganisms; however, they can identify only a small proportion of microorganisms capable of growing under laboratory conditions. Molecular approaches enable comprehensive microbial profiling but are frequently semi-quantitative in nature.

Among molecular methods, 16S rRNA gene sequencing provides a detailed overview of the composition and diversity of bacterial and archaeal communities, including uncultured microorganisms. Nevertheless, this approach is susceptible to amplification bias and generally provides relative rather than absolute abundance data. Quantitative PCR offers sensitive and accurate absolute quantification of specific microbial targets but is limited to known microorganisms and requires appropriate standards for accurate interpretation. A comparative overview of culture-based, molecular-based, and quantitative approaches for rumen microbial community analysis is presented in Figure 5.

Metagenomics represents one of the most comprehensive approaches because it can simultaneously reveal microbial taxonomic composition and potential genetic roles. However, metagenomic analysis remains expensive, requires complex bioinformatics workflows, and typically does not provide absolute quantification unless integrated with complementary quantitative methods. Therefore, contemporary rumen microbiome research frequently combines culture-based, molecular, and quantitative approaches to obtain a more comprehensive and reliable understanding of the rumen ecosystem (Aldridge et al., 2016; da Silva-Marques et al., 2018; O'Hara et al., 2023; Prakash et al., 2023).

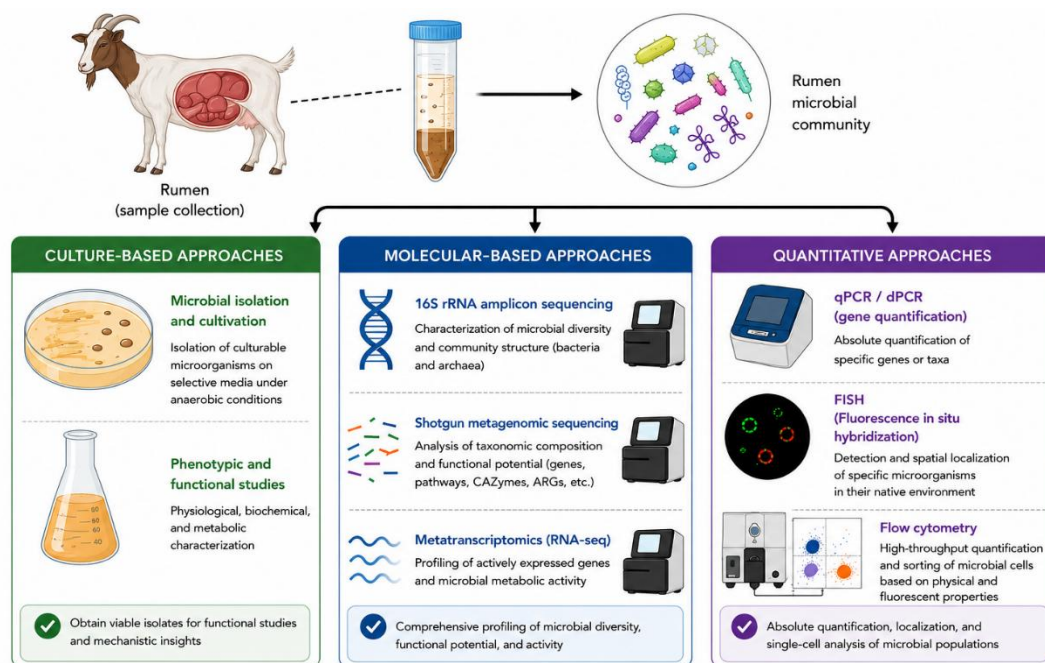


Figure 5. Comparative overview of culture-based, molecular-based, and quantitative approaches for rumen microbial community analysis. Adapted from Deng et al. 2007; Kong et al. 2010; Seshadri et al. 2018; Li et al. 2018).

CONCLUSION

In conclusion, each method used in rumen microbiome research possesses distinct and complementary advantages as well as inherent limitations. The integration of multiple methodological approaches is

increasingly employed in contemporary rumen microbiome research to obtain a more comprehensive, accurate, and reliable understanding of the rumen ecosystem.

AUTHOR CONTRIBUTIONS

Rahayu Wulan conceptualized the study and drafted the manuscript. Nur Hidayah revised and edited the manuscript. All

authors read, approved, and agreed to the published version of the manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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