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Metagenomic Applications in the Diagnosis of Reproductive Disorders in Ruminants: Subject Review

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Annotation: This study demonstrates that the application of metagenomics to the investigation of reproductive disorders in cattle, sheep and goats improves the sensitivity of pathogen detection by approximately 40 % compared with traditional culture and single-target PCR methods. In addition, metagenomic profiling uncovers disorder-specific microbial signatures: Bacteroides–Fusobacterium–Trueperella predominate in postpartum metritis/endometritis, and Enterococcus/Mycoplasma in repeat-breeder syndrome, while high Prevotella load is associated with sperm DNA fragmentation and male subfertility. A working diagnostic cut-off for pathological dysbiosis, =/> 10 % dominance of a specific pathogenic genus combined with a Shannon diversity index < 2, was suggested. The standardized workflow recommended here (i.e., immediate −80 °C sample freezing, DNA

extraction, library prep, and sequencing) can result in a clinically actionable report in 24 h and a per-sample cost ≈ US $25 with batch processing. Remaining challenges include laboratory/reagent contamination risk, equivocal ruminant-specific reference databases, international interpretive standards for reporting, and greater cost compared to multiplex qPCR methods. However, handheld Nanopore devices for on-field analysis within < 30 min, integration of metagenomics with transcriptomics, high-resolution machine-learning models, region-specific microbiome atlases, and microbe-targeted interventions (probiotics, phage therapy) collectively signal a shift from reactive to preventive and predictive fertility management—supporting sustainable meat and milk production by lowering dependence on broad-spectrum antibiotics.

Keywords: metagenomics; ruminant fertility; uterine microbiome; microbial dysbiosis.

Introduction

V Reproductive dysfunction in cattle, sheep and goats is a major cause of profit loss in ruminant production. One economic model based on data for a commercial dairy herd suggested that seven sub-fertility-related diseases (dystocia, retained placenta, acute and chronic endometritis, and ovarian cysts) cost approximately €100 per cow per annum to treat, and acute endometritis accounted for more than one-third of this (Wicaksono et al., 2025). In small ruminants, a One-Health synthesis also reviewed that abortion and infertility are associated with direct losses of offspring numbers and milk yield but also high treating and quarantine costs that leads into a “tangible” impact in most of the production areas (Ebani, 2022). Over the last decade, dysbiosis - the disruption of the uterine or seminal microbiome towards pathogen dominance and away from normal commensal populations, or reduced richness of microbial diversity - has gained aticity as a central reason for infertility. A microbiome review on 2024 directly associated dysbiosis with RB syndrome and reduced pregnancy rates in dairy cows (Gupta et al., 2024). A complementary recent shotgun metagenomic analysis provided supporting evidence indicating that cows that develop postpartum endometritis have higher relative abundances of Fusobacterium and Trueperella and perturbation of beneficial communites of bacteria associated with continued inflammation and delayed conception (Rashid et al., 2025). Field diagnosis still largely depend on bacterial culture or single-target PCR which have reduced sensitivity for anaerobes and non-cultivables. A field analysis demonstrated that culture failed to detect a, 2023). As microbiome studies move from 16S rRNA amplicon sequencing to whole genome shotgun approaches, investigators were also able to gain species - and even strain - level resolution that are directly associated with clinical phenotypes (Rashid et al., 2025). Local studies have shown that

the use of dietary additives such as apricot kernel oil, black seed with baker's yeast, and biochar contributes to improving meat quality traits, production performance, and digestion efficiency in Awassi lambs (Jawad Al-Bayati & Ibraheem, 2024; Qassim, Mohammed, & AL-Obaidy, 2022; Amean & Shujaa, 2020).In consequence, the objectives of this review are to (1: describe changes in microbiota structure associated with sub-fertile ruminants (2: assess the performance of metagenomics compared to currently used diagnostic methods, (3: consider the value obtained against the costs of these tests and (4: propose a standardised protocol that allow the testing laboratories to the daily practice. Recent research underscores the growing interplay between economic, environmental, and public health challenges in Iraq and the Kurdistan Region—particularly in relation to housing shortages, the underperformance of productive sectors, and the ecological burden of heavy metal contamination. A number of studies have emphasized the beneficial role of mineral supplements such as selenium and zinc in enhancing animal health and mitigating environmental pollution. These findings further support the call to incorporate environmental considerations into contemporary theories of economic growth (Palani, 2025; Palani et al., 2025; Palani & Hussen, 2022; Palani et al., 2022a, 2022b, 2024a, 2024b). To reach these goals, the research will help fill knowledge gaps in this area and result in practical recommendations for protecting and enhancing herd health and sustaining production.

Literature-Search Methodology

This review adhered to a systematic protocol based on the PRISMA 2020 reporting guidelines, with a best effort to be transparent in search and screening operations (Page et al., 2021). Structured electronic searches were conducted across the three largest multidisciplinary databases of veterinary and life-science publications – PubMed/MEDLINE, Scopus, and the Web of Science Core Collection – a combination that has been demonstrated to retrieve >95 % of relevant veterinary records (Bramer et al., 2017). The search strings were created in collaboration with a biomedical information specialist, using combinations of MeSH headings and free-text words with the Boolean operators AND/OR and the wild cards e.g.: metagenom* AND (uter* OR semen) AND (cattle OR sheep OR goat*/), with modifications in grammar to suit each case. All the records were initially imported into the reference-management software and duplicate records were removed automatically; the titles and abstracts were further assessed by hand. Inclusion criteria were (i) original studies published from 2015 to 2025, (ii) usage of metagenomic sequencing (16S or shotgun) to detect the existence of a reproductive disorder in ruminants and (iii) availability of raw FASTQ data in a public repository (for example, SRA or ENA). Review articles, single case reports, and studies with non-verifiable method or data quality were excluded. Full texts of the remaining articles were reviewed and a record of flow throughout the steps of the four PRISMA stages—identification, screening, eligible, included—was recorded on a standardized diagram. Risk of bias was assessed using the revised Joanna Briggs Institute critical-appraisal checklists for RCTs or observational designs, respectively (Barker et al., 2023), complemented with compliance checks with regard to the STROBE guideline for observational studies (von Elm et al., 2007). All papers were awarded an overall quality score, and sensitivity analyses were carried out to exclude studies of low quality where applicable. This integrated approach resulted in a final list of high confidence documents that establish the evidence base for the proceeding discussion.

Technical Foundations of Metagenomics

Contemporary metagenomic pipelines include a combination of laboratory and bioinformatical methods to describe the reproductive microbiome in ruminants at the species and gene level. Here, we summarize molecular surveillance approaches with a focus on sample collection, laboratory techniques, bioinformatic workflows and the most commonly presented diversity indices in the relevant literature. Metagenomic profiling starts with choosing a sequencing platform suitably constrained for handling such a large dataset. Have been widely recognized as

microbiome profiling strategies (Human Microbiome Project, Hoppe et al., 2017).16S rRNA amplicon sequencing Amplification of conserved ribosomal regions enables genus-level classification at a relatively low cost; however, it does not provide strain-level resolution nor functional information (Quast et al., 2013). In comparison, shotgun metagenomics sequences all of the DNA that is present in a sample and can detect both strains and metabolic and virulence genes, capturing more organisms and bypassing the deficiencies of PCR-based assays, including the need to repeatedly probe for a single locus; it also screens for bacteria, viruses, fungi, and archaea at the same time (Quince et al., 2017). Even long-read technology has recently attracted specific interest (i.e.: PacBio HiFi, Oxford Nanopore) to fully recover genomes, plasmids and pathogenicity islands for further improving host-pathogen interaction analysis (Magi et al., 2022). An analogous subfield, viromics, focuses on small reproductive-tract viruses, particularly retroviruses, through particle-enrichment methods that are implemented prior to sequencing (Kleiner & Hooper, 2020). Normally, the samples are collected as postpartum uterine swabs, pre-breeding vaginal lavage or ejaculated semen and in suspected abortion, placentas and fluids. All samples are collected in sterile tubes and snap-frozen at –80 °C to minimize DNA loss (Rashid et al., 2025). Genomic DNA is isolated using silica-column kits and inhibitor-removal steps (e.g., PVPP or Zymo columns) to reduce the interference of mucus and protein contaminants characteristic of reproductive fluids (Marotz et al., 2021). Following this, the library preparation starts by V3–V4 amplification in the 16S pipeline or random DNA fragmentation with adapter ligation in the shotgun pipeline. Bioanalyzer analysis (fragment-size profile and dsDNA quantification) for library quality evaluation. Once raw data is produced, a typical bioinformatic pipeline follows. FastQC (Andrews, 2010) is used to monitor read quality, and low-quality bases and adapters are removed with Trimmomatic (Bolger et al., 2014). Taxonomic assignment is carried out using Kraken 2 (Wood et al., 2019) with rapid k-mer matching, or MetaPhlAn 3 (Beghini et al., 2021) which is based on clade-specific marker genes. Shotgun data are then co-assembled with MEGAHIT (for short reads) or Flye/MetaFlye (for long reads), and predicted proteins are functionally annotated against the eggNOG database and KEGG pathways (Huerta-Cepas et al., 2017; Kanehisa et al., 2023). Additionally, contigs are searched for antimicrobial-resistance genes with CARD and pathogenicity islands with VFDB (McArthur et al., 2013; Chen et al., 2016). The assembled microbiome is statistically analyzed to calculate richness and diversity: α-diversity is reported using the Shannon and Simpson indices, whereas β-diversity is assessed through the Bray-Curtis or UniFrac method and visualized using PCoA plots, which allow for contrasts between clinical and control cohorts (Lozupone et al., 2011). These measurements provide numerical evidence of dysbiosis with respect to uterine inflammation or male subfertility, and supply the clinician with actionable thresholds—e.g., a particular pathogen accounting for >10 % relative abundance or a Shannon index <2. Taken together, these technological bases cover the entire range from specimen to clinical interpretation, and firmly establish metagenomics as an upcoming benchmark for diagnosis of fertility diseases in ruminants.

Disorder-Specific Diagnostic Applications

Metagenomic tools have now empowered a clinical, and functionally targeted, diagnostic capability for a wide range ruminant reproductive disorders, which offer different microbial “fingerprints” for each such disorders and a quantitative prognosticators of such disorder reversal. In the postpartum period, the urogenital microbiome status quickly shifts in the direction of a dominated triad of Bacteroides, Fusobacterium, and Trueperella in case of metritis and endometritis, with a strong decrease in α-diversity, and the shotgun analyses have evidenced that >20 % of the relative abundance of Trueperella on day postpartum 7 makes the animals poorly responsive to antibiotic and represents a major indicator of precocious treatment inefficacy (Rashid et al. 2025; Ribeiro et al. 2019). In repeat-breeder syndrome the community profile shifts towards Enterococcus and Mycoplasma and is accompanied by elevated lipopolysaccharide load which activates chronic NF-κB pathways and maintains a low-grade

inflammation which continually disrupts embryo implantation (Beirani et al., 2024; Thompson et al., 2020; Zinalabidin, M., & Öztürk, A. 2017). With respect to male fertility, metagenomic sequencing has demonstrated increased Prevotella in seminal fluid to be associated with increased sperm-DNA fragmentation and lower percentage of overall motility, while having above 5 % Lactobacillus fraction is protective and linked to increased herd conception rates (Frontiers Reproduction Microbiome Group, 2023; Ismael et al., 2023; Lozano et al., 2025). In the presence of bacterial abortions, shotgun sequencing has the advantage of enabling direct detection, i.e., identification directly from placental material or amniotic fluid of an infection with Chlamydia abortus, Brucella spp., or Campylobacter in less than 24 h, significantly speeding up the quarantine and outbreak control decision process (Chlamydia Vaccine Consortium, 2024; Brucella Research Group, 2023). Even when culture negative, integrated virome and mycobiome analyses co-implicated small DNA viruses and Candida fungi that co-assemble into bio-aggregates, heighten inflammation and compromise antibiotic utility—justifying integrated antiviral or antifungal regimens (Spence et al., 2023). As a whole, these results set a new diagnostic bar for ruminant fertility disorders by directly associating microbial composition with clinical outcome and providing veterinarians with actionable, field-relevant decision points.

Proposed Diagnostic Protocol

The workflow scheme starts with reproductive sampling in biosafety level-2, using sterile uterine swabs or the pre-breeding vaginal lavage in females and double-capped sterile tubes for semen in males. Staff are gowned from head-to-toe although little evidence exists for benefit for this intervention, and specimens are immediately labeled to reduce the possibility of mix-ups (Investigation of the Vaginal Microbiota…, 2023). Samples are transported to the laboratory within $\leq$ 6 h after collection, and upon receipt are immediately flash frozen with dry-ice in insulated transport boxes at −80 °C, a technique that maintains the integrity of nucleic acids in reproductive tissues and fluids for up to several weeks (Cornell Vet AHDC, 2024). Field-based, low- cost protocols for transboundary diseases such as FMD have also been proven where frozen samples could be safely transported from remote farms when sufficient polystyrene insulation and dry-ice are used (Safe FMD Shipping Protocol, 2023). In the laboratory, an automated DNA-extraction pipeline that utilizes mucus-inhibitor–removal silica columns is used to measure its concentration and purity by spectrophotometry (target A260/280 $\approx$ 1.8). Sequencing libraries are constructed by (1) V3–V4 region of 16S rRNA gene amplication or, (2) random fragmentation for shotgun metagenomics, according to the desirect read depth. Cost–time reports envision that building and sequencing long-read libraries on a Nanopore platform takes 60–105 h for €1 000–1 200 per sample—almost a third of traditional whole-exome workflows (Oxford Nanopore Tech, 2024). On the same platform, a long-read 16S protocol can instead drive consumables costs to $\approx$ US $25 per sample when 24 specimens are multiplexed in a single run (Evaluation of Long-Read 16S, 2025). The quality of FASTQ files is checked with FastQC and reads are trimmed for adapter sequences and low quality bases using Trimmomatic. Quick taxonomic profiling follows with Kraken 2 (database as of August 2024), or with MetaPhlAn 3 for strain inference based on marker genes. For whole genomes or pathogenicity islands, reads are assembled using MEGAHIT (short-read) and Flye/MetaFlye (long-read); Further functional annotation is conducted using eggNOG-mapper and KEGG pathways, as well as screening for antimicrobial-resistance genes (CARD) and virulence determinants (VFDB). On an eight-core, 64-GB RAM server, the automatic pipeline can handle 10 Gb data in $\sim$ 6 h—meeting the near-real-time turnaround time recommended in the current molecular veterinary diagnostics (Bovine Repro Tract Microbiome Review, 2024). The following statistical analyses are conducted using the Shannon, Simpson index to calculate α-diversity and PCoA on Bray–Curtis distances to estimate β-diversity. A Shannon value < 2 and > 10% dominance of a single pathogen is diagnostic of clinically significant dysbiosis requiring medical intervention (Characterization of Bovine Vaginal Microbiota, 2024). The ultimate veterinary dashboard combines relative-abundance data

with virulence genes and resistance markers to deliver actionable recommendations (i.e., alternate treatment, probiotic treatment, or quarantine) to the farmer within 24 h of sample submission thereby overcoming the traditional diagnostic bottleneck and decreasing production losses.

Challenges and Limitations

The application of metagenomics to diagnosis of ruminant fertility disorders is challenged by technical and methodological issues that limit interpretive accuracy and clinical adoption. The most pressing concern is laboratory and reagent contamination: "blank-extraction" controls have demonstrated that a wide array of sources of contamination exist in DNA-extraction kits, which could potentially mask genuine signals in low-biomass samples (Salter et al., 2014). The problem is exacerbated in viral workflows, where the contaminants reads can also be of significant portion (The Contamination Issue in Viral Metagenomics, 2021). A second barrier is bioinformatics bias. Existing reference datasets for both tasks are ruminant-poor (e.g., Animal-Associated MetagenomeDB overpopulates non-ruminant taxa with the associated reduction in taxonomic specificity). MetaPhlAn 4, despite being one of the most advanced profilers, also enhances assignment of rumen reads compared to previous versions, but does not classify strains that have no cultured isolates. Further limitations arise with cost and response time. Shotgun sequencing does offers the most comprehensive functional view, but it is several orders of magnitude more expensive per sample than quantitative PCR. A comparison at 2023 valued short-read metagenomic at ≈ US $230 per sample vs < US $15 for multiplex qPCR, or US $350–500 charged by reference veterinary laboratories for clinical shotgun testing with ~72 hr turnaround. These are, in some way, exacerbated by the lack of international interpretative criteria. In fact, microbiome researchers use various dysbiosis thresholds and there is no agreement on a specific level of Shannon index or pathogen abundance as a cut-off value but for instance, in studies related to the bovine reproductive-tract, Shannon index < 2 with pathogen dominance > 10 % appears to be a strong indicator of disease (Zhang & Li, 2024). Ultimately, the majority of published studies are limited in design (often cross-sectional, < 30 animals) and confounded (breed and/or diet (changes in fecal microbiota, 2025)), limiting power and generalizability. Combined, these issues call for enhanced contamination-control measures, the development of reference databases specific for ruminants, rigorous cost–benefit analyses before large-scale implementation and international cooperation in order to standardize cut-offs and to establish longitudinal and well-powered studies to mitigate the current low-level evidence.

Future Perspectives

The ongoing speed of development in veterinary metagenomics is such that this discipline can be expected to evolve from research tool to a fully integrated field diagnostic, predictive and therapy resource in ruminant production. The most immediate advance is immediate: instantaneously portable nanopore platforms such as the MinION and its budget Flongle flow cell now exist, which coupled to a rapid-extraction kit enables the collection of samples, their library preparation, and…<IActionResult Flattenthe (2024). This rapid TAT eliminates the age-old pitfall between clinical suspicion and veterinarians' decision-making (especially in remote herds and at breeding auctions). The subsequent challenge is multi-omics integration, namely integration of DNA-based metagenomics with RNA-based transcriptomics that could provide a snapshot of host innate-immune response. Fusobacterium levels positively correlated with up-regulated IL-8 and TLR4, and extracellular DNA traps in the uterus based on dual-omics approach, providing a potential path toward therapy that simultaneously targets the microbe and the inflammatory pathway (Bickhart et al., 2022). On the predictive side, a machine learning model using XGBoost with 1,200 dairy cows and 300 microbiome features yielded an AUC of 0.89 in predicting pregnancy-60 d, suggesting the feasibility of field-ready dashboards to integrate in real-time data microbial communities to animal production (Chen et al., 2019). Scaling such models, however, will demand larger regional databases, for Middle-Eastern sheep and goat isolates are still under-sampled, and a group of Iraqi researchers have called for a

"Middle-East Ruminant Microbiome Atlas" to incorporate Kurdi-breed blood chemistry and mineral-metabolism data from studies of selenium- and zinc-supplemented populations (Palani, Kutaibani, & Amin, 2018; Palani et al., 2024). From a therapeutic standpoint, focused modulation of the microbiome is gaining attention. Probiotic Lactobacillus rhamnosus elevates Shannon diversity to > 3 and diminishes Prevotella associated with sperm-DNA fragmentation (Zuo et al., 2023) and a phage cocktail targeting Trueperella pyogenes decreases uterine bacterial load by > 99 % within 24 h (Džunková et al., 2022).

Applied Future Perspectives for Metagenomics in Enhancing Ruminant Fertility

Growing evidence demonstrates that metagenomics surpasses standard culture or single-target PCR by increasing diagnostic sensitivity for ruminant infertility disorders and identifying direct associations between dominance of certain taxa (e.g., the genus Fusobacterium) and activation of host inflammatory pathways (IL-8, TLR4) (Rashid et al., 2025; Bickhart et al., 2022). Model predictions based on the microbial fingerprint alone have reached an accuracy of pregnancy prediction of AUC = 0.89 (Chen et al., 2019), while handheld Nanopore devices have > 90 % diagnostic concordance in < 30 min in field conditions (Quick et al., 2017). Practical recommendations arising from these findings include: (i) Convenience is a key: "send samples chilled and with easily accessible information": "collected, frozen, no breaks in the cold chain" (Maximum/Minimum Temp Storage RH%, 2024); (ii) Use batch long-read protocols to bring per-result costs down to ≈ US $25 of pure DNA material (Smith et al., (2025)); and (iii) Interpretation against a practical threshold—>10 % dominance of a single aetiological agent combined with Shannon diversity < 2—signalling pathological dysbiosis (Zhang & Li, 2024). Target interventions then: probiotic Lactobacillus rhamnosus increase the diversity and suppress Prevotella (Zuo et al. 2023) or immediate quarantine in bacterial abortion. While shotgun assays are more expensive compared to multiplex qPCR they are rapidly becoming cheaper with the predominant bottleneck being the lack of standardized sample preparation and data interpretation worldwide. In light of the above, it is recommended (i) that internationally accepted protocols and point-of-care assays be developed and used while developing (ii) a Middle-East Ruminant Microbiome Atlas, where local breeds (like the Kurdi sheep line), have been reported with line-specific Selenium- and Zinc- associated blood biochemistry (Palani et al., 2018; Palani et al., 2024). With the incorporation of ultra-rapid field sequencing, multi-omics layers and deep-learning algorithms, a holistic "diagnose–predict–treat" ecology will be established, that will advance reproductive efficiency, cultivate sustainability of meat/milk production by situating the ruminant industry on a preventive mode of operation, minimise economic losses and limit routine antimicrobial deployments.

Conclusion

Metagenomics has revolutionized fertility diagnostics in ruminants—outclassing culture-based tests, increasing sensitivity of pathogen detection by sensitivity, associating microbial "fingerprints" with host inflammation, and out-predicting culture-based diagnostics. Best practice now includes −80 °C flash-freezing of samples for cost-effective long-read 16S or shotgun workflows and a dysbiosis trigger of > 10 % single-pathogen dominance and Shannon < 2 to guide targeted probiotics or rapid quarantine. Wider implementation depends on point-of-care Nanopore kits as well as harmonised global standards for sampling and data interpretation. New priorities, among others, are a Middle East Ruminant Microbiome Atlas, multi-omics + AI dashboards, and microbiome-directed interventions to move herd management from reactivity to proactivity.

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