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A Review of Metagenomic Studies on Microbial Diversity and Fermentation Profiles in Total Mixed Ration Silage

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Abstract
Total Mixed Ration (TMR) silage plays a vital role in modern livestock nutrition by providing a balanced and complete feed source. Microbial communities present during the ensiling process profoundly influence silage fermentation quality, nutrient preservation, and ultimately animal performance. Traditional culture-based methods have limited resolution in characterizing these complex microbial ecosystems, whereas metagenomic approaches, including 16S rRNA gene sequencing and whole-genome shotgun sequencing, offer comprehensive insights into microbial diversity and functional potential. This review synthesizes current metagenomic research on TMR silage, highlighting the dominant roles of lactic acid bacteria, yeasts, and other microbes in fermentation dynamics. Key factors affecting microbial succession, such as moisture, temperature, oxygen exposure, and inoculation strategies, are discussed alongside their impact on fermentation profiles and silage stability. Comparative analyses underscore the greater microbial complexity and resilience in TMR silage compared to single-forage silages. Despite advancements, metagenomic methods face challenges like limited taxonomic resolution, sequencing biases, and difficulty distinguishing active microbes, which can be overcome through integration with complementary omics approaches such as metatranscriptomics and metabolomics. Future applications of multi-omics promise enhanced precision in silage management, tailored inoculants, and improved livestock productivity. Overall, metagenomics offers a powerful toolset for optimizing TMR silage quality and supporting sustainable animal agriculture.
Keywords: Total mixed ration, metagenomics, lactic acid bacteria, fermentation

Introduction

A total mixed ration (TMR) can be categorized as a complete feed, as it contains all the necessary nutrients for livestock animals. The TMR is a blend of concentrates, forages, vitamins, minerals, additives, and byproducts (Šístkova et al., 2015). Ensiling TMR has been a practice since the 1960s, and recently, a lot of research has been done to improve the formulation of TMR silage. It is crucial to analyze the metagenomic study of microbial diversity in TMR as it has a huge role in the silage fermentation and quality (Bueno et al., 2020).

Numerous studies have shown that the nutrient composition and fermentation characteristics are directly influenced by the microbes present during the fermentation phases. Traditional microbiological approaches for metagenomic studies often rely on culture-based techniques, and this technique has limited ability as it is unable to provide accurate bacterial diversity. While molecular techniques are able to provide deeper information compared to the traditional techniques (Xie et al., 2020).

Utilizing metagenomics in studying silage microbiology is crucial due to its ability to provide a comprehensive understanding of microbial communities during the fermentation process. On the other hand, metagenomics also allows for direct sequencing of DNA extracted from silage samples. This encourages the identification of both cultivable and non-culturable microbial species present in silage.

This kind of detailed analysis cannot be obtained from traditional approaches. A metagenomic study is able to help the ruminant industry by enhancing the feed quality and nutritional value. A study by Mikolajczyk in 2020 emphasizes how specific plant additives such as Lactobacillus plantarum and Lactobacillus buchneri influence the methanogenesis and volatile fatty acid (VFA) synthesis in the rumen, as it connects the microbial community composition in silage with its physiological effects on ruminants. This highlights the importance of understanding microbial interactions through metagenomics. Hence, this paper aims to review recent advances in metagenomic applications for TMR silage, focusing on microbial diversity, functional roles, factors influencing microbial succession, challenges, and future perspectives in optimizing silage quality and livestock productivity.

Principle of Metagenomics in Silage Research

Several advanced techniques, such as 16S rRNA sequencing and whole genome shotgun sequencing, provide a robust framework for understanding microbial diversity and ecology. Each of these methods possesses unique advantages and limitations that contribute to this microbial study. The 16S rRNA sequencing method focuses on amplifying and sequencing a specific region of the RNA gene, which is highly conserved across bacterial species. By amplifying and sequencing these regions, research can identify and classify bacterial taxa present in silage samples. This enables researchers to identify the closely related bacterial species. However, utilizing the 16S rRNA sequencing technique is limited by its relatively low taxonomic resolution and inability to reveal the metabolic potential of the microbial communities.

A study by (Matchado et al., 2024) emphasized that functional profiles from 16S rRNA gene data are generally not suitable to discern metabolic capabilities, recommending more advanced techniques like shotgun metagenomics for functional insights. The study showed that while 16S sequencing can sometimes give a rough idea of certain functional genes in ideal situations, the tools used to interpret these results are usually not sensitive or accurate enough to reliably identify important functional changes (Wensel et al., 2022). This is especially true for functions related to human health issues like colorectal cancer, obesity, and type 2 diabetes. These problems occur partly because of technical challenges, such as differences in the number of 16S rRNA gene copies between microbes and gaps in the reference genome databases. Even after improving data processing and utilizing better tools, the limited level of detail provided by 16S sequencing still makes it challenging to accurately predict the functions of microbial communities.

In contrast, whole-genome shotgun sequencing (WGS) involves the random sequencing of all DNA present in a sample, enabling the capture of genetic information from all microorganisms such as bacteria, fungi, archaea and viruses. This method offers detailed information not only about the composition of microbial communities but also their functional capabilities, enabling researchers to better understand microbial ecology and metabolic interactions in environments like silage. The WGS offers several advantages. It provides high-resolution data that can distinguish between different strains within the same species, allowing a deeper understanding of microbial interactions at a finer scale than 16S rRNA sequencing (Byrd et al., 2020). Additionally, WGS can reveal the full range of functions within microbial communities, which is especially important when studying complex ecosystems or evaluating how the microbiome affects health (Wang et al., 2020).

Despite its advantages, whole genome sequencing (WGS) is more expensive and requires more powerful computational resources for data analysis compared to 16S rRNA sequencing. Additionally, the complexity involved in piecing together genomes from metagenomic data can make it challenging to interpret the functions of identified genes and understand how they impact the overall microbial community. Hence, both 16S rRNA sequencing and whole-genome shotgun (WGS) sequencing are essential tools in metagenomics. The choice between them depends on the research goal: 16S rRNA sequencing is ideal for identifying microbial taxa with good taxonomic resolution, while WGS provides a deeper understanding of microbial functions and overall diversity. Integrating these approaches continues to advance our knowledge of microbial ecosystems, supporting improvements in areas such as silage fermentation, agriculture, medicine, and environmental management. Together, they offer a comprehensive framework to better understand and optimize the microbial communities crucial to TMR silage.

Microbial Diversities in TMR Silage

The TMR silage is a fundamental feeding practice in livestock production. It provides a complete nutrient profile required by animals and supports a diverse microbial community that plays a crucial role in fermentation and in determining silage quality. The microbial composition in TMR silage can significantly influence the efficiency of fermentation, stability during storage and nutritional value of feed. Key microbial groups in TMR silage include lactic acid bacteria (LAB), yeasts, and various other microbes, each contributing uniquely to fermentation and influencing the overall quality of the silage.

The dominant group of microorganisms in TMR silage is the lactic acid bacteria (LAB), which are pivotal for maintaining anaerobic conditions for the quality fermentation process. Creating an acidic environment within the silage is important to suppress the undesirable microbes. The LAB, such as *Lactobacillus plantarum* and *Lactobacillus casei,* not only enhance silage quality but also influence nutritional characteristics, such as vitamin preservation (Zhao et al., 2022).

Yeast is also present in significant amounts in TMR silage. Studies show that genera such as Cryptococcus and Pichia are major contributors to facilitate the fermentation process and enhance the nutritional profile of silage through various metabolic pathways such as amino acid metabolism, carbohydrate metabolism, etc. These yeasts, like *Cryptococcus* and *Pichia*, play a helpful role in the fermentation of silage by breaking down nutrients and improving its overall quality. However, if their numbers get too high, they can turn against the process and cause spoilage. This imbalance can lead to nutrient loss and reduce the feed's value, so it's important to keep their populations in check to ensure the silage stays fresh and nutritious.

The bacterial and fungal diversity within TMR silage is largely determined by the ensiling process and the composition of the original feed ingredients. For instance, the microbial community present in fresh TMR, predominantly consisting of *Firmicutes* and *Bacteroidetes*, differs significantly from that found in the silage after fermentation and storage. The ensiling conditions, characterized by low oxygen and increased acidity, reduce overall microbial diversity by favouring the survival of microorganisms better adapted to these harsh environments. This selective pressure results in a simplified microbial community, particularly among LAB. The reduced diversity of LAB underscores their critical role in ensuring a successful fermentation process and producing silage of high quality.

Factors Influencing the Microbial Dynamics

The changes in microbial populations during silage fermentation are shaped by several interconnected factors, such as moisture levels, temperature, exposure to air, storage conditions, and the use of microbial inoculants or additives. Gaining a clear understanding of these dynamics is essential for improving fermentation results and producing high-quality silage, which plays a crucial role in maintaining good livestock nutrition.

Moisture content is one of the most critical factors affecting microbial dynamics in silage. The dry matter (DM) content (30-40%) directly influences the fermentation process and microbial succession patterns. Optimum moisture content has been linked to improved preservation of nutrients and increased microbial activity. For instance, Valle et al. noted that microbial inoculants can support optimal moisture levels (60-70%) in silage, which can enhance fermentation product generation, such as organic acids, contributing positively to silage quality (Del Valle et al., 2018).

Storage temperature plays a crucial role in shaping the microbial communities in silage. According to Bai et al., (2022), higher storage temperatures, around 30°C, can significantly alter the bacterial composition and fermentation characteristics. They observed that silage stored at this elevated temperature experienced a rapid drop in pH within the first 12 hours, which negatively impacted microbial diversity. (L. Li et al., 2022) highlighted that changes in temperature have a significant impact on the quality of fermentation. They found that lower temperatures tend to result in less effective fermentation and higher levels of leftover nitrates. Their research showed that fermentation performed notably better at a warmer temperature of 25°C compared to 10°C, reinforcing the importance of maintaining suitable temperatures during ensiling to promote optimal microbial activity and improve fermentation efficiency.

Oxygen exposure can harm silage quality by encouraging the growth of aerobic bacteria, which leads to spoilage. Therefore, maintaining strict anaerobic conditions during the ensiling process is essential to prevent the spread of unwanted microbes. If oxygen is present before the silage is properly sealed, it can significantly change the microbial community, promoting the growth of undesirable organisms (Xu et al., 2019). Moreover, the methods used for storage play a critical role in shaping

microbial dynamics and composition, as effective sealing and proper storage practices help reduce losses caused by aerobic spoilage (Hooker et al., 2019).

Inoculants play a key role in improving silage fermentation by promoting the rapid growth of beneficial lactic acid bacteria (LAB). This leads to a quicker reduction in pH and better overall fermentation results. For example, Meng et al., (2025) demonstrated that adding *Lactiplantibacillus plantarum* to soybean silage notably enhanced both fermentation quality and nutritional value, highlighting the effectiveness of targeted inoculation. Additionally, the use of certain additives like chitosan has been found to support microbial growth, suggesting that combining inoculants with such additives can further improve silage quality (Del Valle et al., 2018). The author also mentioned that an increase in crude protein (CP) of about 5.75 g/kg and energy content of approximately 3.0 g/kg in inoculated silages, implying enhanced nutritional value through improved microbial activity.

Fermentation Profiles and Their Microbial Drivers

The fermentation process in silage production generates several important end-products that have a significant impact on both the quality of the silage and the health of the animals consuming it. These compounds, including organic acids, alcohols, and gases, result from the activity of various microorganisms during ensiling. Among them, lactic acid, acetic acid, and propionic acid stand out as the primary fermentation products, each playing a distinct role in shaping silage characteristics and supporting livestock nutrition.

Lactic acid is the main product of effective fermentation, predominantly produced by LAB. It plays a vital part in lowering the pH of silage, creating an acidic environment that effectively inhibits harmful spoilage organisms and pathogens. Research by Mendonça et al., (2020)demonstrated that silages with higher lactic acid content tend to have greater aerobic stability and overall quality, which results in reduced nutrient losses and enhanced animal productivity. Similarly, Li et al., (2022)highlighted that improved fermentation quality closely aligns with higher lactic acid levels, showing direct benefits for animal health through better nutrient availability. Furthermore, Kuppusamy et al., (2020)emphasized that efficient lactic acid fermentation by specific LAB strains not only preserves silage but also enhances its digestibility and nutritional value, thereby supporting improved livestock performance. Collectively, these findings underline lactic acid's critical contribution both in protecting silage from spoilage and in boosting animal growth and productivity.

Acetic acid, produced primarily through heterolactic fermentation, also contributes to silage stabilization. It helps maintain a lower pH and plays an important role in preventing mould development, particularly when silage is exposed to oxygen during feed-out. Huang et al., (2021)observed that silages with a well-balanced ratio of lactic and acetic acids typically exhibit superior fermentation quality due to their combined antimicrobial effects against undesirable microbes. Propionic acid is another valuable fermentation product known for its ability to inhibit spoilage organisms, thereby enhancing the storage durability of silage. (Mendonça et al., 2020) and Zhang et al., (2019)further reported that the use of specialized microbial inoculants can increase acetic and propionic acid levels, improving silage stability during both storage and feeding phases. This demonstrates the potential benefits of carefully tailored microbial supplements to optimize fermentation end-products and improve silage outcomes.

The fermentation end-products present in silage have a direct influence on its nutritional value and, consequently, on animal health. Ferreira et al., (2023) pointed out that a higher proportion of lactic acid relative to other acids is a strong indicator of high-quality fermentation, which translates into better nutrient digestibility and overall health benefits for livestock. Conversely, the detection of butyric acid, a marker of poor fermentation, is associated with reduced feed intake and lower digestibility, negatively affecting animal performance (Oliveira et al., 2016). Beyond nutrition, the fermentation process can also impact environmental and animal health by influencing methane emissions during ruminal digestion. Mikołajczyk et al.,(2020) reported that silage quality affects methane production in the rumen, highlighting that optimizing fermentation not only improves feed quality but can also contribute to reducing greenhouse gas emissions from livestock.

Comparative Study between TMR silage and Single-Forage Silage

The TMR silages, which are made up of a mix of different forage types and concentrate ingredients, naturally host more complex microbial communities. The varied composition of TMR creates a range of microenvironments that can support a wider variety of microorganisms compared to silages made from a single forage type. While this complexity may result in somewhat more variable fermentation results,

it also offers enhanced resilience against spoilage organisms, as the diverse microbial populations can compete with and inhibit unwanted microbes, helping to maintain silage quality.

A comprehensive meta-analysis by Oliveira et al., (2017)systematically reviewed multiple studies examining the effects of lactic acid bacteria (LAB) inoculation on silage fermentation and animal performance. Their findings consistently demonstrated that inoculating silage with LAB significantly improves fermentation quality in both Total Mixed Ration (TMR) and single-forage silages. This improvement is primarily due to the rapid establishment of beneficial LAB populations that accelerate lactic acid production, reduce pH more quickly, and inhibit the growth of spoilage microorganisms such as clostridia and enterobacteria. Consequently, these improvements help minimize nutrient losses during ensiling, preserve dry matter, and enhance the overall nutritional value of the silage.

Moreover, the author also highlighted that the benefits of LAB inoculation extend beyond fermentation metrics to positive impacts on animal health and productivity. Improved silage quality correlates with increased feed intake, better nutrient digestibility, higher milk yield, and enhanced growth performance in livestock. This underscores the critical role of microbiological management in optimizing silage outcomes and the importance of selecting effective inoculants tailored to the forage type and ensiling conditions.

Challenges and Future Directions of Metagenomic Study

Metagenomics has indeed revolutionized the characterization of silage microbial communities by facilitating culture-independent profiling, allowing for a comprehensive examination of microbial taxa and their associated functional capabilities. However, the current methodologies in metagenomics present several limitations that affect their efficacy. Most notably, techniques such as 16S rRNA amplicon sequencing and shotgun metagenomics possess unique constraints regarding taxonomic resolution. While 16S rRNA sequencing may effectively identify microbial communities, it often fails to capture strain-level diversity and functional gene discrepancies, potentially missing significant microbial interactions integral to silage fermentation processes. In contrast, shotgun metagenomics offers greater taxonomic resolution, yet it necessitates substantial sequencing depth to accurately characterize complex microbiomes, which can lead to increased costs (Durazzi et al., 2021).

Furthermore, biases in DNA extraction and PCR amplification are critical concerns as they impact the representation of microbial communities, particularly those taxa that exist in low abundance (Nies et al., 2021). It has been documented that certain DNA types, such as those from GC-rich regions, may be underrepresented, complicating downstream analyses (Nogueira & Botelho, 2021). Although metagenomics can suggest the presence of various genes, it often lacks the capability to delineate active metabolic pathways or specific microbial functions without supplementary data. Moreover, existing annotation databases may lack completeness, thus hindering precise functional attributions of identified sequences. One significant challenge remains the inability of metagenomic techniques to distinguish between DNA from living versus inactive microbes, leading to potential confounding factors in the interpretation of microbial roles during silage fermentation (Nies et al., 2021).

To address these limitations, there is a growing consensus on the integration of metagenomics with complementary "omics" approaches, which collectively provide a more holistic understanding of silage microbial ecosystems. For instance, meta-transcriptomics, which sequences community RNA instead of DNA, allows for the capture of gene expression profiles, pinpointing actively functioning microbes and their metabolic pathways throughout the fermentation stages. Additionally, the profiling of both volatile and non-volatile metabolites offers crucial insights into fermentation products and microbial activities, correlating these outputs with key attributes of silage quality, including pH and nutrient preservation (Yulandi et al., 2020). Proteomics can further enhance this understanding by identifying expressed microbial proteins, elucidating the enzymatic functions and metabolic activities present during fermentation.

Incorporating multi-omics strategies is particularly promising for improving silage quality and yielding better livestock outcomes. Identifying beneficial microbial species and their functional roles could inform the creation of targeted, strain-specific inoculants aimed at optimizing fermentation efficiency, enhancing aerobic stability, and improving nutritional content. Moreover, the development of metagenomic tools to enable real-time monitoring of silage microbial communities presents an innovative approach for detecting spoilage organisms early, allowing for timely interventions to curtail potential losses (Yulandi et al., 2020). By manipulating the microbial consortia within silage to promote

pathways that generate advantageous organic acids or break down anti-nutritional factors, livestock nutrition and digestibility can be enhanced significantly.

While metagenomics has undoubtedly transformed the landscape of silage microbiology, addressing its current methodological limitations through the integration of various "omics" is essential to fully unlock insights into microbial ecology. These advancements pave the way for precision interventions that not only bolster silage preservation but also support animal nutritional needs and contribute to overall environmental sustainability in the realm of livestock farming.

Conclusion

In conclusion, metagenomic studies have significantly advanced our understanding of the complex microbial communities responsible for fermentation in TMR silage. These approaches reveal how crucial lactic acid bacteria and other microbial groups are in interacting and responding to environmental factors, such as moisture content, temperature, and oxygen levels, to influence silage quality and stability. The integration of molecular techniques, especially combining 16S rRNA and whole-genome shotgun sequencing, allows for more detailed insights into microbial diversity and functional potential compared to traditional culture-dependent methods. Nonetheless, current metagenomic strategies are limited by technical constraints, including taxonomic resolution and sequencing biases, which can be effectively addressed by integrating complementary multi-omics technologies like metatranscriptomics and metabolomics. Leveraging these comprehensive data will enable the development of targeted microbial inoculants, real-time monitoring tools, and precision fermentation management techniques. Such advancements are essential to improve silage preservation, enhance nutrient availability, boost animal health and productivity, and promote environmentally sustainable livestock farming practices. Continued research into the microbial ecology of TMR silage through innovative omics approaches will be pivotal in meeting the growing demands of efficient and sustainable animal production systems.

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