Research Advances in the Discovery and Application of Silage Microorganisms: Postprint

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Abstract

The species and abundance of silage microorganisms play a crucial role in the silage fermentation system, and exploring and utilizing beneficial silage microorganisms represents a hot spot and key focus in this research field. This review summarizes the recognition and identification of novel silage microbial species, common types and mechanisms of action of silage microbial inoculants, and their effects on silage quality, aerobic stability, and ruminant production performance, aiming to provide technical approaches and theoretical foundations for developing high-quality silage microbial inoculants, improving silage nutritional quality, and enhancing ruminant production performance.

Full Text

Research Progress on Discovery and Utilization of Silage Microbes

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Abstract

The types and abundances of silage microbes play a key role in the silage fermentation system. Exploring and utilizing beneficial microbes in silage represents a hotspot and priority in this research field. This paper reviews recent advances in the identification of novel silage microbial species, common types of silage microbial inoculants and their mechanisms of action, and the effects of these inoculants on silage quality, aerobic stability, and ruminant production performance. The aim is to provide technical approaches and theoretical foundations

for developing high-quality silage microbial inoculants, improving silage nutritional quality, and enhancing ruminant production performance.

Keywords: silage; microbiology; metagenomics; inoculants; aerobic stability

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Statistics indicate that over 60% of China' s annual corn grain consumption is used for feed, yet the resulting corn straw is not efficiently utilized in livestock production, leading to low conversion efficiency and utilization difficulty of crop byproducts as feed resources. Considering the comprehensive utilization of resources, pollution reduction, and value-added product development, the integrated utilization of crop straw is essential [1]. With the strategic adjustment of agricultural structure and rapid development of animal husbandry in China, vigorously promoting the cultivation of whole-plant silage corn and developing a "planting-feeding-planting" model integrated with ruminant farming, along with actively implementing cost-reduction and efficiency-enhancing projects such as the "grain-to-feed" initiative, have become important measures to address feed resource shortages and the competition for grain between humans and livestock. To improve ruminant production performance and better convert whole-plant corn and alfalfa into high-quality feed, utilizing silage microbial fermentation technology and adding beneficial microbes to improve silage quality represents a crucial strategy [2]. This paper summarizes the current status and latest research progress on silage microbial development and utilization both domestically and internationally, reviewing silage microbial technology, species, the mechanisms by which microbial inoculants affect silage, nutritional quality, aerobic stability, and animal production performance, thereby providing theoretical foundations and practical references for further improving silage quality and ruminant production performance.

1. Understanding and Identification of Novel Silage Microbial Species

Traditional culture methods combined with modern molecular biotechnology, such as PCR-denaturing gradient gel electrophoresis (DGGE), 16S rRNA high-throughput second-generation 454 pyrosequencing, third-generation single-molecule sequencing, metagenomics, and metatranscriptomics, enable rapid and accurate detection of microbial diversity and community structure [2-6]. Han et al. [7] used PCR-DGGE to detect microbial communities in total mixed ration silage and identified *Lactobacillus acetotolerans* and *L. pontis* strains. Li et al. [8] employed high-throughput sequencing to monitor changes in over 30 bacterial genera during the silage process. Liu [9] applied Miseq high-throughput sequencing to assess microbial diversity in switchgrass silage after 60 days. Tao et al. [10] combined laboratory testing with Miseq high-throughput sequencing to analyze silage quality while providing information on microbial community composition and abundance changes before

and after ensiling, thereby offering a basis for fermentation process regulation. Bao et al. [11] utilized third-generation single-molecule sequencing technology to examine microbial changes in alfalfa silage and their impact on quality, demonstrating its utility for evaluating silage microbial dynamics and quality. Our research group employed Miseq high-throughput sequencing to analyze microbial dynamics and community structure changes during whole-plant corn silage fermentation and aerobic exposure, finding that short-term aerobic exposure increased the proliferation of harmful *Clostridium* bacteria [12].

With the integration of traditional cultivation and continuously evolving PCR-based technologies, novel microbial species in silage are being continuously discovered and utilized. These new strains are isolated, screened, and cultured for inoculation into silage, after which methods such as PCR-DGGE and high-throughput sequencing-metagenomics are used to elucidate their roles in silage fermentation. This represents an effective approach for discovering and utilizing novel silage microbes.

In recent years, microbial species isolated from silage are summarized in . Understanding the growth and fermentation characteristics of these novel microbial species allows for screening beneficial strains as silage microbial inoculants while identifying harmful microbes as references for developing antimicrobial strategies.

Table 1 Microbial species isolated from silage

Microbial group	Species (Latin name)	Silage type	Reference
Lactic acid bacteria	*Lactobacillus fructivorans*	Alfalfa	Wu et al. [13]
	Lactobacillus plantarum	Grassland forage	Wang et al. [14]
	Lactobacillus plantarum	Grass silage	Valan et al. [15]
	Lactobacillus mixtipabuli	Total mixed ration silage	Tohno et al. [16]
	Lactobacillus acetotolerans	Corn silage	Li et al. [17]
	Lactobacillus panis	Corn silage	Li et al. [17]
	Lactobacillus reuteri	Corn silage	Li et al. [17]
	Lactobacillus taiwanensis	Silage	Wang et al. [18]
	Lactobacillus zeae	Corn silage	Rossi et al. [19]
	Lactobacillus silagincola	Silage	Tohno et al. [20]
	Lactobacillus pentosiphilus	Silage	Tohno et al. [20]

Microbial group	Species (Latin name)	Silage type	Reference
	Leuconostoc lactis	Corn stover	Pang et al. [21]
	Enterococcus flavescens	Corn silage	Brusetti et al. [22]
	Enterococcus mundtii	Corn stover	Pang et al. [21]
	Paralactobacillus selangorensis	Italian ryegrass	Parvin et al. [23]
	Pediococcus dextrinicus	Italian ryegrass	Parvin et al. [23]
	Pediococcus parvulus	Corn silage	Li et al. [24]
	Weissella cibaria	Corn, corn stover	Pang et al. [21]
	Weissella kimchii	Corn silage	Brusetti et al. [22]
	Weissella paramesenteroides	Corn silage	Li et al. [24]
Anaerobic spore formers	*Clostridium baratii*	Corn silage	Rossi et al. [19]
	Paenibacillus macerans	Corn silage	Rossi et al. [19]
Bacillus	*Bacillus megaterium*	Corn silage	Brusetti et al. [22]
Enterobacteria	*Erwinia persicina*	Italian ryegrass	Li et al. [25]
	Pantoea agglomerans	Italian ryegrass	Li et al. [25]
	Rahnella aquatilis	Italian ryegrass	Li et al. [25]
Acetic acid bacteria	*Acetobacter pasteurianus*	Corn silage	Li et al. [17]
Yeast	*Saccharomyces martiniae*	Corn silage	Li et al. [24]
	Pichia deserticola	Corn silage	Li et al. [24]
	Pichia kudriavzevii	Corn silage	Li et al. [24]
	Pichia fermentans	Corn silage	Rossi et al. [19]
	Candida apicola	Corn, ryegrass	Rossi et al. [19]
	Candida intermedia	Corn silage	Li et al. [24]
	Candida glabrata	Corn silage	Li et al. [24]
	Candida magnolia	Corn silage	Li et al. [24]

	Candida mesenterica	Corn silage	Rossi et al. [19]

Wu et al. [13] isolated *Lactobacillus fructivorans* from wilted alfalfa silage. Wang et al. [14] isolated five lactic acid bacterial strains from Hulunbuir grassland silage, including *Lactobacillus plantarum*, *Pediococcus dextrinicus*, *L. brevis*, *L. casei*, and *L. graminis*, with *L. plantarum* showing the strongest acid production, fastest fermentation rate, and superior acid and low-temperature tolerance, making it suitable as a silage additive for the Hulunbuir region. Wang et al. [18] isolated and identified a novel species named *Lactobacillus taiwanensis* from silage. Rossi et al. [19] surveyed farm silages, primarily corn, alfalfa, ryegrass, and their mixtures, identifying the novel species *Lactobacillus zeae* in corn silage. Among anaerobic spore-forming bacteria, most were known *Clostridium* species except *Clostridium baratii* and *Paenibacillus macerans*. Three yeast species were identified: *Candida mesenterica*, *C. apicola*, and *Pichia fermentans*, with *C. mesenterica* and *C. apicola* being reported for the first time. Brusetti et al. [22] used LH-PCR to identify *Bacillus megaterium* (at 0 and 1 day), *Weissella kimchii* (at 6 days), and *Enterococcus flavescens* (at 13 days) during early-stage corn silage fermentation, representing the first reports of these species in silage.

2.1. Common Types and Mechanisms of Silage Microbial Inoculants

Silage microbial inoculants are typically classified into four categories: (1) homofermentative lactic acid bacteria that rapidly reduce pH and increase lactic acid bacteria populations and lactic acid content; (2) obligate heterofermentative lactic acid bacteria that primarily enhance aerobic stability; (3) facultative heterofermentative lactic acid bacteria that combine both functions; and (4) other microbial inoculants, or non-lactic acid bacteria (N-LAB), such as novel combinations of *Streptococcus bovis* with homofermentative LAB, *Propionibacterium*, *Bacillus*, and yeast. These inoculants primarily promote fermentation, inhibit aerobic deterioration, and improve nutrient content and digestibility [23].

To compensate for insufficient epiphytic microbes on silage materials (less than 10 CFU/g fresh matter) and better promote silage fermentation, inoculants should be added to ensure fermentative bacteria populations of at least 10 CFU/g fresh weight, accelerating pH reduction and transition into the fermentation phase. The mechanism involves creating an anaerobic environment where appropriate microbial inoculants combine with native epiphytic LAB to accelerate fermentation, enhance rapid LAB proliferation, and quickly reduce pH below 3.8. The resulting organic acids, particularly lactic acid, effectively inhibit other microorganisms, minimizing nutrient loss, improving energy conversion efficiency, maintaining fresh green color and aroma, and enhancing palatability for ruminants [2]. Therefore, adding suitable inoculants during silage fermentation can accelerate the process, improve silage quality, enhance utilization

efficiency, increase aerobic stability, and effectively boost animal feed intake.

2.2. Effects of Silage Microbial Inoculants on Silage Quality and Aerobic Stability

Over the past two decades, microbial inoculants have been widely applied in most regions worldwide, with the majority being homofermentative LAB inoculants such as *Lactobacillus plantarum*, *L. casei*, *Enterococcus faecium*, and *Pediococcus* species. The primary purpose of these additives is to rapidly and efficiently produce lactic acid during fermentation, enabling faster pH reduction and minimizing dry matter and crude protein losses to preserve nutritional value and quality. However, research has shown that these products can negatively affect the aerobic stability of whole-plant corn silage, likely due to reduced acetic acid content. Currently, there is an urgent industry need to identify a homofermentative LAB inoculant that can produce organic acids as the main fermentation product while maintaining silage stability.

Heterofermentative LAB belong to the *Lactobacillaceae* family, including *Lactobacillus buchneri*, *L. reuteri*, *Oenococcus*, *Leuconostoc*, and *Weissella*. *Lactobacillus buchneri* is widely used as a representative microbial inoculant. This strain grows slowly and can produce acetic acid from sugars or lactic acid even after fermentation completion. Increased acetic acid inhibits yeast and mold growth, delaying silage spoilage and improving aerobic stability. However, this inoculant has limitations, including slower growth compared to homofermentative LAB and requiring 50–70 days of ensiling to improve aerobic stability. Therefore, further research is needed on specific strains and inoculation rates of *L. buchneri* to overcome these deficiencies [25]. Recent studies indicate that a novel *L. buchneri* strain A KKP 2047p can convert 1,2-propanediol to propionic acid, which contributes to glucose and vitamin B12 production [26], though whether naturally occurring strains possess this capability remains unclear and requires further investigation.

Combined application of *L. buchneri* with traditional inoculants can improve both silage quality and animal performance while enhancing aerobic stability. Guo et al. [27] used single-molecule real-time (SMRT) sequencing and metabolomics to examine bacterial community dynamics and metabolic profiles in alfalfa silage inoculated with homofermentative *L. plantarum* and heterofermentative *L. buchneri*, providing bioinformatics insights into microbial changes during silage fermentation and offering regulatory methods for producing different quality silages through various inoculant types.

Combined application of facultative and obligate heterofermentative LAB represents an important approach to achieve dual benefits. Facultative heterofermentative LAB control harmful bacteria such as *Clostridium* during fermentation, reducing crude protein hydrolysis and dry matter loss. Obligate heterofermentative LAB, primarily *L. buchneri*, slowly convert lactic acid to acetic acid during late fermentation, increasing pH and aerobic stability, thereby improving animal

daily gain and milk production. However, it remains unclear whether feeding silage with combined inoculants can consistently enhance animal performance. In vitro digestion studies have shown that silage can improve fiber digestibility [28], though whether similar benefits occur in ruminants requires further in vivo verification.

Regarding novel inoculants, *Streptococcus bovis* exhibits similar functions to LAB in silage. This species was identified and screened from the rumen rather than silage environments and reproduces rapidly, doubling every 20 minutes—faster than typical silage LAB. Ferreira et al. [29] added two *S. bovis* strains to elephant grass silage for 60 days, finding that compared to untreated and *E. faecium*-inoculated silages, *S. bovis* reduced pH and ammonia nitrogen content while decreasing gas and effluent losses, thereby increasing dry matter content. These strains show potential as inoculants for tropical forage silage. *Bacillus subtilis* is a newly developed strain that improves aerobic stability by producing bacteriocins inhibitory to yeasts and molds, with applications in corn and alfalfa silage [30]. Recently, yeasts such as *Pichia* and *Saccharomyces cerevisiae* have been reported as novel silage inoculants [31], showing potential to inhibit harmful microbes and reduce aerobic losses, though controlling their growth in silage applications remains challenging for commercialization. Some newly discovered inoculants produce antimicrobial and antimycotic compounds that inhibit various harmful bacteria and molds in silage, providing valuable microbial resources for improving silage quality and reducing harmful substances during storage and utilization [30-31].

2.3. Effects of Silage Microbial Inoculants on Ruminant Production Performance

While silage microbial inoculants positively affect silage quality and can improve animal production performance, the underlying mechanisms remain unclear. In some cases, silage quality shows clear relationships with animal performance, though mostly indirect, and improvements in dry matter digestibility may correlate with enhanced production. Ando et al. [32] found that adding *Lactobacillus rhamnosus* to guinea grass silage improved dry matter and organic matter digestibility, increasing feed intake in castrated rams.

Recent studies indicate that inoculated silage can improve in vitro rumen fermentation. In vitro research on rice whole mixed ration silage inoculated with *L. plantarum* showed reduced methane production compared to untreated silage [33]. Ellis et al. [34] reported that adding LAB as probiotics or fermentation starters increased in vitro rumen digestibility while decreasing methane production. Daniel et al. [35] inoculated corn silage with a mixture of homofermentative *L. lactis*, *L. plantarum*, and *E. faecium*, which improved total mixed ration digestibility and increased milk yield in dairy cows. These results suggest that some inoculants can alter rumen fermentation by either reducing methane production or increasing microbial biomass, both potentially enhancing animal performance. For ruminant producers, improving production efficiency is the

primary goal of adding microbial inoculants, yet the effects on production efficiency remain poorly understood. Therefore, research on silage inoculants' effects on ruminant performance should focus on screening strains that enhance rumen microbial activity rather than solely reducing fiber content during ensiling. Integrating novel PCR-based technologies, metabolomics, and in vitro fermentation analysis [27, 36-37] will facilitate deeper investigation into how silage inoculants affect ruminant utilization and production performance.

4. Conclusions and Outlook

Research on silage feed in China started relatively late, particularly regarding the development and application of silage microbial inoculant products, creating a substantial gap with developed countries. This disparity is incompatible with China's "grain-to-feed"initiative and the transition of ruminant production from extensive to intensive systems. In recent years, rapid development in China' s dairy, beef, and sheep industries has created demand for high-quality silage, yet backward silage technology and lack of quality microbial inoculants have become major constraints on ruminant production and cost-efficiency improvements. As demand for silage quality increases and chemical additive use decreases, selecting non-toxic, harmless, inexpensive, and convenient microbial inoculants represents the trend for China' s silage industry development.

Although foreign silage microbial inoculants have been applied for many years and entered the Chinese market, China lacks independently developed, high-quality products, necessitating strengthened research and development efforts. We must employ traditional cultivation combined with modern molecular biotechnologies—particularly continuously updated techniques such as 16S rRNA high-throughput third-generation single-molecule and whole-genome sequencing, metagenomics, metabolomics, and transcriptomics—to continuously identify novel microbial species in silage resources and isolate superior inoculants. This will provide theoretical foundations and new application strategies for further improving silage quality and ruminant production performance.

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Note: Figure translations are in progress. See original paper for figures.