Evaluation of Aerobic Stability of Smooth Bromegrass Silage Fermented with Grass-Derived Homofermentative and/or Heterofermentative Lactic Acid Bacteria: Postprint

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Abstract

This study aimed to evaluate the effects of grass-derived homofermentative and/or heterofermentative lactic acid bacteria fermentation on the aerobic stability of Bromus inermis silage. Two homofermentative strains, Lactobacillus plantarum and Enterococcus mundtti, and one heterofermentative strain, Lactobacillus buchneri, isolated and screened from grasses were prepared as inoculants. Flowering-stage Bromus inermis was harvested and cut into 2~3 cm lengths. Four treatments were established based on the inoculant applied: 1) control treatment (con), sprayed with sterile deionized water; 2) heterofermentative treatment (he), sprayed with Lactobacillus buchneri suspension; 3) homofermentative treatment (ho), sprayed with a mixed suspension of Lactobacillus plantarum and Enterococcus mundtti (mixed at a 1:1 ratio); and 4) combined homofermentative and heterofermentative treatment (he+ho), sprayed with a mixed suspension of Lactobacillus buchneri, Lactobacillus plantarum, and Enterococcus mundtti (mixed at a 1:1:1 ratio). The application rate for all treatments was 10 mL/kg fresh forage, with a total bacterial count of approximately $5\$\times 105CFU/gfreshforage, andeachtreatmenthadfivereplicates.After60doffermentationatroomtemperatu ensiling(P < 0.05), andalltreatmentsexhibitedgoodfermentationquality(pH\ \$4.3)$, particularly the ho treatment. During the aerobic exposure test, the pH values of the con and ho treatments increased rapidly, exceeding 7 by day 8; the pH increase in the he+ho treatment was slightly lower than that in the con and ho treatments, reaching 6.3 by day 8, which was significantly lower than the con and ho treatments (P<0.05); the pH increase in the he treatment was very slow, reaching only 4.4 by day 8, which was significantly lower than the other treatments (P<0.05). After 60 d of ensiling, the water-soluble carbohydrate (WSC) content was highest in the ho treatment, significantly higher than in the other treatments (P<0.05), and lowest in the con treatment, significantly lower

than in the other treatments (P<0.05). During the aerobic exposure test, WSC content decreased rapidly in the ho treatment and slowly in the he treatment; by day 8, WSC content was highest in the he treatment, significantly higher than in the he+ho treatment (P<0.05), while the con and ho treatments were significantly lower than both the he and he+ho treatments (P<0.05). Mold counts were significantly suppressed after ensiling, with the suppression effect being superior when heterofermentative lactic acid bacteria were added alone or when homofermentative and heterofermentative lactic acid bacteria were added in combination; no molds were detected in these two treatments, and the he+ho treatment still showed no molds by day 5 of aerobic exposure, while the he treatment showed no molds by day 8 of aerobic exposure. On days 3 and 5 of aerobic exposure, yeast counts in the he and he+ho treatments were significantly lower than those in the con and ho treatments (P<0.05). On day 8 of aerobic exposure, the lactic acid and acetic acid concentrations in the he+ho and he treatments were significantly higher than those in the other two treatments (P<0.05). The aerobic stability of the he, he+ho, con, and ho treatments decreased sequentially, being 194, 126, 62, and 58 h, respectively. Based on the comprehensive evaluation results, it was concluded that inoculation with Lactobacillus buchneri alone or in combination with homofermentative lactic acid bacteria Enterococcus mundtti and Lactobacillus plantarum could effectively inhibit aerobic spoilage of Bromus inermis silage and ensure quality stability during aerobic exposure, with the former being more effective; inoculation with homofermentative lactic acid bacteria Enterococcus mundtti and Lactobacillus plantarum did not demonstrate a positive effect in improving the aerobic stability of Bromus inermis silage.

Full Text

Evaluation of Aerobic Stability of Bromus inermis Leyss. Silage Fermented by Homofermentative and/or Heterofermentative Lactic Acid Bacteria from Grass

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Abstract

This study was conducted to evaluate the effects of homofermentative and/or heterofermentative lactic acid bacteria (LAB) isolated from grasses on the aerobic stability of Bromus inermis Leyss. silage. Two homofermentative LAB strains (Lactobacillus plantarum and Enterococcus mundtii) and one heterofermentative LAB strain (Lactobacillus buchneri) were isolated from grasses and

prepared as inoculants. Bromus inermis Leyss. was harvested at the flowering stage and chopped to 2–3 cm lengths. Four treatments were established based on the inoculant type: 1) control (con), sprayed with sterile deionized water; 2) heterofermentative (he), sprayed with Lactobacillus buchneri; 3) homofermentative (ho), sprayed with a mixture of Lactobacillus plantarum and Enterococcus mundtii (1:1 ratio); and 4) combined heterofermentative+homofermentative (he+ho), sprayed with a mixture of all three strains (1:1:1 ratio). The application rate was 10 mL/kg fresh forage for all treatments, delivering approximately 5$×10^{5}$ CFU/g fresh forage. Each treatment had five replicates. After 60 days of ensiling at ambient laboratory temperature, aerobic stability was evaluated by measuring nutritional composition, microbial populations, and core temperature changes.

The results showed that after 60 days of ensiling, pH values in all treatments decreased significantly compared to pre-ensiling values ($P<0.05$), with all silages exhibiting good fermentation quality (pH$ $4.3), particularly the ho treatment. During aerobic exposure, pH in the con and ho treatments increased rapidly, exceeding 7 by day 8. The he+ho treatment showed a more moderate pH increase, reaching 6.3 on day 8, which was significantly lower than con and ho ($P<0.05$). The he treatment exhibited a very slow pH increase, reaching only 4.4 on day 8, significantly lower than all other treatments ($P<0.05$). After 60 days of ensiling, the ho treatment had the highest water-soluble carbohydrate (WSC) content, significantly greater than other treatments ($P<0.05$), while the con treatment had the lowest. During aerobic exposure, WSC content decreased rapidly in ho but slowly in he. By day 8, the he treatment maintained the highest WSC content, with he+ho being significantly lower ($P<0.05$), and both con and ho being significantly lower than he and he+ho ($P<0.05$).

Mold growth was strongly inhibited after ensiling, with complete suppression in the he and he+ho treatments. The he+ho treatment remained mold-free until day 5 of aerobic exposure, while the he treatment showed no molds even at day 8. Yeast counts in he and he+ho treatments were significantly lower than in con and ho treatments on days 3 and 5 ($P<0.05$). On day 8, lactic and acetic acid concentrations in he and he+ho treatments were significantly higher than in the other two treatments ($P<0.05$). Aerobic stability values were 194, 126, 62, and 58 h for he, he+ho, con, and ho treatments, respectively.

In conclusion, inoculation with Lactobacillus buchneri alone or in combination with homofermentative LAB (Lactobacillus plantarum and Enterococcus mundtii) effectively inhibited aerobic deterioration and maintained quality stability of Bromus inermis Leyss. silage during aerobic exposure, with the single inoculation being more effective. Inoculation with homofermentative LAB alone did not improve aerobic stability.

Keywords: Bromus inermis Leyss.; homofermentative lactic acid bacteria; heterofermentative lactic acid bacteria; silage; aerobic stability

Introduction

During feed-out, air infiltration into silage pits can induce proliferation of aerobic microorganisms (yeasts, molds) that were dormant during the anaerobic phase, causing temperature and pH increases that lead to aerobic deterioration and substantial nutrient losses [1-2]. Therefore, preventing or reducing aerobic spoilage and improving aerobic stability are critical for ensuring silage quality. Current research on aerobic stability typically employs three evaluation approaches: 1) temperature change (aerobic stability is defined as the duration for which the core temperature remains within 2°C of ambient temperature after exposure) [3]; 2) chemical analysis (measuring changes in pH, organic acid content, WSC, dry matter, CO_2 production) [4-5]; and 3) microbial cultivation (monitoring changes in LAB, yeasts, molds, and clostridia) [4-5].

Lactic acid bacteria used in silage production are taxonomically diverse, with different species exhibiting distinct growth characteristics, functions, and roles in the fermentation ecosystem. Homofermentative LAB (hoLAB) are widely used in silage production due to their rapid fermentation, effective inhibition of undesirable microorganisms, and low dry matter losses, which help preserve nutritional value [6-7]. However, recent research on aerobic stability has shown that hoLAB-based inoculants provide little benefit and may even reduce aerobic stability, predisposing silage to aerobic spoilage [8-9]. In contrast, heterofermentative LAB (heLAB) have gained attention for their positive effects on improving aerobic stability [10-11], and the combined application of homofermentative and heterofermentative LAB has become a research focus [12-13].

Under natural conditions, grasses harbor relatively few epiphytic LAB and numerous undesirable microorganisms [14-15]. Successful silage production requires LAB inoculation to dominate fermentation and preserve nutrients. Commercial inoculants are predominantly imported, expensive, and their performance under local conditions requires verification. Different strains exhibit varying characteristics and effects on different silage substrates [16-17]. This study integrated the three evaluation methods to assess the effects of three grass-derived LAB strains with different fermentation types on the aerobic stability of Bromus inermis Leyss. silage.

1.1 Preparation of Lactic Acid Bacterial Inoculants

Three strains isolated from grasses were used: Lactobacillus plantarum, Enterococcus mundtii (both homofermentative), and Lactobacillus buchneri (heterofermentative). A loopful of each strain was transferred to 5 mL MRS broth and incubated at 30°C for 72 h, yielding approximately 1$×10^{9}$ CFU/mL. For inoculant preparation, the required volume of culture was taken (2 mL for single strains, 1 mL each for two-strain mixtures, or 0.7 mL each for three-strain mixtures), diluted tenfold with sterile deionized water, mixed uniformly before spraying, and applied at 10 mL/kg fresh forage to achieve approximately 5$×10^{5}$ CFU/g fresh forage.

1.2 Silage Preparation

Bromus inermis Leyss. was harvested at the flowering stage in Zhenglan Banner, Xilingol League, chopped to 2–3 cm, and sprayed with the prepared inoculant using a hand sprayer. The material was thoroughly mixed, packed into 3 L transparent polycarbonate bottles (~1.5 kg per bottle), compressed, and sealed. Four treatments were established: 1) con (sterile water); 2) he (Lactobacillus buchneri); 3) ho (Lactobacillus plantarum + Enterococcus mundtii); and 4) he+ho (all three strains). Each treatment had five replicates. Silos were fermented at ambient laboratory temperature for 60 days.

1.3 Sample Collection and Measurements

Nutrient composition (dry matter [DM], crude protein [CP], acid detergent fiber [ADF], neutral detergent fiber [NDF], and WSC) was determined at day 0 (pre-ensiling) and day 60. After 60 days, three replicates per treatment were opened, and samples were collected on days 0, 1, 3, 5, and 8 of aerobic exposure (samples were taken sequentially without resealing). For each sample, 30 g fresh silage was soaked in 270 mL distilled water for 60 min, blended, filtered through four layers of cheesecloth, and the filtrate was used for pH and volatile fatty acid (VFA) and lactic acid (LA) analysis. Microbial populations (LAB, molds, yeasts) and WSC content were monitored dynamically during aerobic exposure.

1.4 Analytical Methods

Nutrient composition (DM, CP, ADF, NDF) was analyzed according to Zhang Liying [18]. WSC was determined by anthrone colorimetry [19]. pH was measured directly using a portable pH meter (Horiba Twin-B-212). VFA and LA concentrations were determined by gas chromatography: LA reacts with tetramethylammonium hydroxide to form a quaternary ammonium salt, which is dissolved in dimethylformamide and derivatized with methyl iodide at room temperature. The methyl ester derivatives were separated on a stainless steel column (2 m × 3 mm) packed with 15% dinonyl phthalate and 5% Tween-80 on Chromosorb HP (80–100 mesh) using a Shimadzu GC-9A gas chromatograph (Japan) with flame ionization detection. Operating conditions: column temperature 100°C, detector and injector temperature 150°C; carrier gas (N_2) flow 30 mL/min, H_2 50 mL/min, air 500 mL/min; injection volume 1 L.

Microbial counts were determined by plate counting using MRS agar for LAB, Martin medium for molds, and potato dextrose agar for yeasts, incubated at 35°C for 3 days. Aerobic stability was measured by placing 2.5–3.0 kg of 60-day silage in sterilized plastic buckets at room temperature (23$±$1°C), inserting a thermometer into the geometric center, and recording temperature every 2 h. Aerobic stability was defined as the number of hours before the core temperature exceeded ambient temperature by 2°C [20].

1.5 Statistical Analysis

Data were analyzed using one-way ANOVA in SAS 9.0. Duncan' s multiple range test was used for pairwise comparisons, with $P<0.05$ indicating significant differences and $P<0.01$ indicating highly significant differences.

Results

2.1 Changes in pH, Nutrient Content, and Epiphytic Microorganisms Before and After Ensiling

presents changes in pH, nutrients, and microbial populations. After 60 days, all treatments showed significantly reduced pH ($P<0.05$), decreasing from 6.0 to below 4.3, with ho treatment achieving the lowest pH. Nutrient composition changed variably: DM, NDF, and ADF did not differ significantly between treatments ($P>0.05$). CP content decreased significantly in all treatments compared to fresh forage ($P<0.05$), with the greatest loss in con and the least in he+ho, indicating that both homofermentative and heterofermentative LAB inhibited protein degradation, with the combined treatment being most effective. WSC content decreased significantly during fermentation ($P<0.05$), with the greatest loss in con and the least in ho.

Epiphytic LAB populations increased markedly after ensiling, with greater proliferation in he, ho, and he+ho treatments compared to con. Yeast populations, which were high initially, increased slightly during fermentation, with the greatest increase in ho and the least in he. Molds were abundant initially but were strongly suppressed during fermentation, with no molds detected in he and he+ho treatments.

2.2 Dynamic pH Changes During Aerobic Exposure

shows dynamic pH changes during aerobic exposure. No significant pH changes occurred within 24 h, indicating that low initial pH inhibited spoilage organisms. As exposure continued and organic acids decreased, pH increased substantially after 24 h in con and ho treatments, after 72 h in he+ho, and remained stable throughout in he. On days 3, 5, and 8, pH values in he and he+ho treatments were significantly lower than in con and ho ($P<0.05$), demonstrating that Lactobacillus buchneri inhibited spoilage organism proliferation and improved aerobic stability.

2.3 Dynamic Changes in Organic Acid Concentrations

presents organic acid concentration changes. Lactic acid concentrations declined in all treatments during aerobic exposure, with the sharpest decrease in ho. Although he had the lowest initial lactic acid concentration, it lost only 0.79% DM by day 8, compared to losses of 2.33% DM, 4.01% DM, and 2.25% DM in con, ho, and he+ho treatments, respectively. On day 8, lactic and acetic acid concentrations in he and he+ho were significantly higher than in con and ho

(P<0.05), indicating that Lactobacillus buchneri effectively inhibited lactic acid loss and pH rise.

Acetic acid concentrations also declined during aerobic exposure, with losses of 0.93% DM, 0.45% DM, 0.91% DM, and 0.51% DM in con, he, ho, and he+ho treatments by day 8. He and he+ho treatments maintained significantly higher acetic acid concentrations than con and ho (P<0.05). Propionic acid gradually decreased to undetectable levels in all treatments. Butyric acid was detected at low levels, with slightly higher concentrations in con that also decreased over time.

2.4 Dynamic Changes in Microbial Populations

Microbial population dynamics are shown in . LAB populations remained stable across treatments during aerobic exposure. Yeast populations increased slightly over time, but he and he+ho treatments had significantly lower counts than con and ho on days 3 and 5 (P<0.05), with he showing the lowest counts. Molds were never detected in he, appeared only on day 8 in he+ho, and were consistently present in con and ho, with increasing counts over time in ho.

2.5 Dynamic Changes in WSC Content

WSC content decreased in all treatments during aerobic exposure as microorganisms utilized it for growth. The ho treatment showed the earliest and most dramatic decline. He treatment exhibited minimal WSC loss during the first 5 days, with a noticeable decrease only by day 8. He+ho treatment also showed substantial WSC loss, but the decline began later than in ho. On days 5 and 8, WSC content followed the pattern: he > he+ho > con > ho, with significant differences between most treatments (P<0.05). This demonstrates that Lactobacillus buchneri effectively inhibited aerobic microbial growth and reduced WSC consumption.

[Figure 1: see original paper] Dynamic changes of WSC content in Bromus inermis Leyss. silage after aerobic exposure

2.6 Effects of Different LAB Types on Aerobic Stability

As shown in [Figure 2: see original paper], aerobic stability values were 62, 194, 58, and 126 h for con, he, ho, and he+ho treatments, respectively. The ho treatment was 4 h less stable than con, while he was 132 h more stable, and he+ho was 64 h more stable. These results indicate that Lactobacillus buchneri improved aerobic stability, while the homofermentative strains alone had no positive effect, though the three-strain combination also improved stability.

[Figure 2: see original paper] Aerobic stability of Bromus inermis Leyss. silage in each treatment

Discussion

3.1 LAB Inoculants Dominated Fermentation in Bromus inermis Leyss. Silage

Epiphytic microorganisms affect both silage fermentation and inoculant performance. Freshly cut forage contains diverse microorganisms, with aerobic organisms like molds and yeasts often predominating and providing no benefit to fermentation. Liu [21] reported that epiphytic microorganisms on Elymus spp. were mainly general bacteria, yeasts, and molds, with LAB counts below 5$×10^{4}$ CFU/g FM—insufficient for successful silage fermentation (>10^5 CFU/g FM required). In this study, fresh Bromus inermis Leyss. had only 3.72 log(CFU/g FM) LAB but over 6 log(CFU/g FM) yeasts and molds, necessitating LAB inoculation for quality silage production.

pH is a crucial indicator of silage quality, and LAB inoculation rapidly reduces pH. The final pH reflects the dominant fermentation flora. Enterococcus mundtii grows rapidly in early fermentation, quickly acidifying the silage, while Lactobacillus plantarum is acid-tolerant and continues proliferating at low pH [21], resulting in the fastest pH decline and lowest final pH in ho treatment. As a heterofermentative strain, Lactobacillus buchneri produces weaker acids, yielding higher pH values.

Protein degradation during fermentation causes nutrient loss. LAB inoculation inhibits protease activity through rapid pH reduction [22]. All inoculants in this study inhibited protein degradation, with the combined treatment being most effective. The high WSC content and abundant yeasts/molds in flowering-stage Bromus inermis Leyss. could increase DM loss and reduce stability, warranting evaluation of heterofermentative LAB. Heterofermentative LAB alone or combined with homofermentative strains suppressed mold growth more effectively than homofermentative LAB alone. Although ensiling had limited effect on yeast suppression, heterofermentative treatments showed superior performance. Taylor et al. [23] observed yeast population resurgence in later fermentation stages, reflecting dynamic interactions between LAB and yeasts. Some acid-tolerant yeasts can metabolize lactic acid and WSC at pH <3, while butyric acid can inhibit them [24]. The high yeast counts in later fermentation stages may relate to these mechanisms, though specific species identification requires further study.

3.2 Lactobacillus buchneri Effectively Inhibited Aerobic Deterioration

Lactobacillus buchneri is an obligate heterofermentative LAB that produces high acetic acid concentrations, inhibiting yeast and fungal activity and preventing spoilage during aerobic exposure [25]. Studies on various forages (corn, sorghum, barley, wheat, alfalfa, ryegrass, king grass) have consistently shown that Lactobacillus buchneri improves aerobic stability [26], though the magnitude varies with inoculation rate [23,27], opening time [23,27], and location [28]. In this study, single inoculation with Lactobacillus buchneri (he treatment)

uniquely affected aerobic deterioration indicators: 1) it effectively inhibited lactic and volatile fatty acid losses, maintaining low pH (below 4.5 when others exceeded 6.0); 2) high acetic acid concentrations suppressed aerobic spoilage both at opening and during exposure; 3) yeast proliferation was minimal, with the lowest final counts and no mold detection; 4) WSC consumption was lowest, resulting in significantly higher final WSC content; and 5) temperature rise was delayed longest. These results demonstrate that the grass-derived Lactobacillus buchneri effectively inhibited aerobic deterioration and maintained quality stability.

3.3 Effects of Combined Homofermentative and Heterofermentative LAB Inoculation

Post-exposure changes in silage physicochemical properties reflect spoilage rate and extent, influenced by final pH, LA/VFA concentrations, and aerobic microbial growth. Homofermentative LAB produce more lactic acid and stronger acidification, reducing nutrient losses [29] and ensuring high fermentation quality. However, they reduce antifungal compounds, decreasing aerobic stability [30], and residual lactic acid and WSC provide substrates for aerobic microbes [29]. In this study, the ho treatment, despite having the lowest pH and highest lactic acid at opening, showed rapid lactic acid decline and pH rise during exposure, with minimal acetic acid production, resulting in aerobic stability 4 h lower than con. Thus, the homofermentative strains used did not improve aerobic stability.

Some studies have achieved "dual benefits" of promoting anaerobic fermentation while improving aerobic stability using combined inoculants in high-moisture corn, sorghum, bermudagrass, and ryegrass [13,31]. In this study, the three-strain combination (he+ho) maintained significantly higher lactic and acetic acid concentrations and lower yeast and mold counts than con and ho on day 8, with optimal effects during the first 5 days. Although aerobic stability was lower than he alone, it was significantly higher than con and ho. Therefore, the three-strain combination inhibited spoilage organism growth and improved aerobic stability, though less effectively than Lactobacillus buchneri alone.

Conclusion

Grass-derived heterofermentative Lactobacillus buchneri effectively inhibited aerobic deterioration and maintained quality stability of Bromus inermis Leyss. silage during aerobic exposure. Grass-derived homofermentative Lactobacillus plantarum and Enterococcus mundtii did not improve aerobic stability. The three-strain combination also inhibited spoilage organisms and improved aerobic stability, though less effectively than Lactobacillus buchneri alone.

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