



Article

Improving Total Mixed Ration Silage: Effects of Lactic Acid Bacteria Inoculants and Antimicrobial Additives on Fermentation Quality and Aerobic Stability

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Abstract: This work aimed to assess microbial inoculants (*Lactiplantibacillus plantarum* and *Lentilactobacillus buchneri*), chemical additives (natamycin and hexanoic acid), and their combination on fermentation characteristics and aerobic stability in total mixed ration (TMR) silage. The TMR consisted of 30% water bamboo shell (WBS), 10% alfalfa, 20% rice straw, and 40% concentrate. There were six treatments as follows: (1) deionized water (control, CON). (2) lactic acid bacteria (*Lactiplantibacillus plantarum* + *Lentilactobacillus buchneri*; LPB, 1×10^6 cfu/g FW). (3) natamycin (NT, 0.02 g/kg FW). (4) hexanoic acid (HA, 0.02 g/kg FW). (5) lactic acid bacteria + natamycin (SLNT, 0.02 g/kg FW). (6) lactic acid bacteria + hexanoic acid (SLHA, 0.02 g/kg FW). After fermentation, laboratory silos (10 L) were opened to assess fermentation quality, followed by a 6-day aerobic stability test. The results showed that all silages were well fermented with high lactic acid (LA) content, low ammonia nitrogen (NH₃-N), and negligible butyric acid (BA) levels. Among all silages, SLNT silage exhibited the greatest LA, acetic acid (AA) levels, LAB counts, and the lowest pH and NH₃-N. For aerobic stability, all additives significantly ($p < 0.05$) enhanced aerobic stability, delayed ($p < 0.05$) the decrease in LA and water-soluble carbohydrates (WSC) and the increase in pH, and significantly ($p < 0.05$) minimized yeast proliferation. The SLNT silage showed the best aerobic stability, with SLHA, NT, HA, and LPB following. In conclusion, SLNT is recommended as the optimal additive in improving the fermentation quality and aerobic stability of TMR silage, with SLHA, NT, HA, and LPB following.

Keywords: microbial and chemical additives; total mixed ration silage; fermentation quality; aerobic stability



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1. Introduction

Total mixed rations (TMR) are formulated from roughage, by-products, concentrates, minerals, and vitamins in precise ratios, which can provide adequate nutritionally balanced diets for ruminants [1]. Fewer digestive upsets, off-feed situations, and greater milk production have been reported when feeding ruminants TMR rather than separate ingredients [2]. Unfortunately, the rapid deterioration in TMR requires preparation immediately prior to feeding, which is a challenge for family farms with limited labor and machinery [3]. For this reason, fermented total mixed rations (FTMR) have been proposed as a promising approach, and have been increasingly adopted in recent years [4]. TMR silages offer multiple benefits, including homogeneous composition, enhancing palatability through fermentation-altered odors and flavors of by-products, reducing the requirement of labor and machinery, and a potential eco-friendly approach for waste recycling and lowering feed expenses. The downside of TMR silages is the high risk of aerobic deterioration with its high nutritive

peculiarity, which may promote undesirable microorganisms (such as mold and yeast) rapidly multiplying during feeding after opening the silos. Therefore, the great expectation of this work was to explore an effective, eco-friendly, and safe approach to improving aerobic stability. Microbial and chemical additives were recommended to mitigate feed deterioration to make quality silages [5,6].

Lactiplantibacillus plantarum, a facultative homofermentative lactic acid bacterium, was employed to quickly produce lactic acid, lower pH, inhibit undesirable microorganisms, and lower DM and nutritional losses of silages [7]. Nonetheless, it also increased the risk of aerobic deterioration due to the lack of antifungal volatile fatty acids (VFAs) [8]. Conversely, *Lentilactobacillus buchneri*, a heterofermentative lactic acid bacterium, is recommended to delay aerobic spoilage by transforming water-soluble carbohydrates or LA into antifungal acetic acid (AA), thereby improving aerobic stability during feed-out [9,10]. Therefore, the blend of *L. plantarum* and *L. buchneri* in this work aims to ensure synergistic effects for improving fermentation quality and aerobic stability [11].

Natamycin, a polyene macrolide (bacteriocin), is sourced from *Streptomyces natalensis*, which attaches to the ergosterol of mold and yeast membranes without disrupting the plasma membrane, thereby preventing yeast aerobic spoilage during the early stage of ensiling and aerobic exposure [12]. Moreover, natamycin as a safe antifungal feed additive has received approval in Europe due to its minimal intestinal absorption and complete excretion in feces [13]. Woolford et al. [14] were the first to evaluate natamycin and confirmed its effectiveness in inhibiting yeast growth during aerobic exposure. Hexanoic acid, a medium-chain fatty acid, shows strong antifungal activity by disrupting the cell membrane, altering pH, and disturbing osmotic balance and respiratory processes, leading to the destruction of bacterial cells [15]. Hexanoic acid can inhibit yeast growth and delayed aerobic deterioration in high-moisture Italian ryegrass silage [16]. To the best of our knowledge, few works have simultaneously compared LAB (*L. plantarum* and *L. buchneri*), natamycin, hexanoic acid, and their combination in improving TMR fermentation quality and aerobic stability. We hypothesized that microbial inoculants (*L. plantarum* and *L. buchneri*), chemical additives (natamycin and hexanoic acid), and their combination would enhance fermentation quality and aerobic stability.

This work aimed to assess LAB (*L. plantarum* and *L. buchneri*), natamycin, hexanoic acid, and their combination on fermentation characteristics and aerobic stability in TMR silage.

2. Material and Methods

2.1. Total Mixed Ration (TMR) Silage Preparation

The TMR consisted of 30% water bamboo shell (WBS), 10% alfalfa, 20% rice straw, and 40% concentrate on an FW basis. WBS was collected from Zhejiang, China on 10 October 2019. Alfalfa, rice straw, and concentrate were sourced from a mid-sized family farm (Zhejiang, located at latitude 29.43° N, longitude 121.48° E, and an elevation of 4 m). The concentrate includes 8% crushed shelled corn, 25% corn peel, 27% soybean meal, 20% whole cottonseed, 15% wheat bran, and 5% vitamin–mineral. All roughages were cut into 2–3 cm pieces using a sterile cutter. Table 1 shows the chemical and microbial compositions in TMR. The work utilized a completely randomized design as detailed below:

- (1) Deionized water (control, CON).
- (2) Lactic acid bacteria (*Lactiplantibacillus plantarum* + *Lentilactobacillus buchneri*, LPB).
- (3) Natamycin (NT).
- (4) Hexanoic acid (HA).
- (5) Lactic acid bacteria + natamycin (SLNT).
- (6) Lactic acid bacteria + hexanoic acid (SLHA).

Lactiplantibacillus plantarum and *Lentilactobacillus buchneri* strains were supplied by the Institute of Ensiling and Processing of Grass department at Nanjing Agricultural University, China, both with a targeted total inoculation rate at 1×10^6 cfu/g FW. The natamycin and hexanoic acid were food-grade additives with a specified purity exceeding 99%, applied to 20 mg/kg FW. LPB, NT, HA, SLNT, and SLHA were diluted with deionized

water to a specified concentration, then evenly spread in TMR at 5 mL/kg FW. CON was treated with an equal volume of deionized water. The 6 kg TMR for each treatment was placed into silos with a capacity of 10 L, a diameter of 27.5 cm, and a height of 31.6 cm, with approximately 600 kg DM/m³. In total, 120 silos (fermentation quality: 6 treatments × 5 replicates + aerobic stability: 6 treatments × 5 replicates × 3 test time points) were stored at room temperature. Five silos per treatment were opened and sampled to assess fermentation quality after 45 days of anaerobic fermentation. The remaining silos underwent a 6-day aerobic stability experiment.

Table 1. Chemical and microbial compositions of total mixed ration.

Items ¹	Mean
Chemical compositions (g/kg DM)	
Dry matter (g/kg FW)	585
Crude protein	141
WSC	83.9
Neutral-detergent fiber	462
Acid-detergent fiber	243
Ash	100
Ether extract	60.2
BC (mEq/kg DM)	186
Microbial compositions (log₁₀ cfu/g FW)	
Lactic acid bacteria	6.51
Aerobic bacteria	6.31
Yeasts	5.09

¹ DM: dry matter; FW: fresh weight; WSC: water-soluble carbohydrate; BC: buffer capacity; Log₁₀: decimal logarithm. cfu: colony-forming unit.

2.2. Chemical Composition and Fermentation Quality Analysis

The buffering capacity of TMR was determined according to Playne and McDonald [17]. Three subsamples were prepared from the TMR silages.

The first subsample (200 g) was blended with distilled water (600 mL) and subjected to extraction at 4 °C for 24 h. Subsequently, the extract was passed through four layers of cheesecloth and a filter paper for filtration. The filtrate was utilized to measure pH, ammonia nitrogen (NH₃-N), organic acids, and ethanol. The pH was measured with a pH meter (Hanna Instruments Inc., Woonsocket, RI, USA). The NH₃-N content was measured following Broderick and Kang [18]. The organic acids and ethanol contents were determined using an Agilent 1260 HPLC system (Agilent Technologies, Inc., Waldbronn, Germany). The total digestible nutrients (TDN) were calculated as follows [19]:

$$TDN (\%) = 82.75 - (0.704 \times ADF) \quad (1)$$

The second subsample of TMR or TMR silage was dried (65 °C, 48 h) in an oven to measure the DM content following the specified method of Wang et al. [20]. The dry sample was then ground and passed through a 1 mm screen using laboratory knife mills (93ZT-300; Xingrong Co., Ltd., Guangzhou, China) for the analysis of water-soluble carbohydrates (WSC), total nitrogen (TN), neutral-detergent fiber (NDF), acid-detergent fiber (ADF), ether extract (EE), and ash. The WSC content was determined following the specified method of Arthur Thomas [21]. The TN content was measured using a Kjeltac 8200 Auto-Analyzer (FOSS Analytical AB, Höganäs, Sweden) and the CP content was determined using $TN \times 6.25$. The NDF and ADF contents were analyzed following the specified method of Van Soest et al. [22]. The EE and ash contents were measured following the Association of Official Analytical Chemists standard procedures AOAC [23]. The non-fibrous carbohydrate (NFC) was determined according to Council [24]. The third subsample of TMR or TMR silage was used for microbial counts following the specified method of Wang, Liu, Zhao, Dong, Li, and Shao [20].

2.3. Aerobic Stability Test

A total of 15 silos per treatment were opened and mixed homogeneously after 45 days of anaerobic fermentation, and then transferred into 15 L sterile polyethylene buckets (30 cm diameter \times 35 cm height). The opened silos were covered with gauze and kept at room temperature. The six probes of a multichannel temperature recorder (MDL-1048A) were positioned at various points within the bottles to record temperatures every 30 min over a period of 6 days. Additionally, six probes were positioned in the ambient environment as a blank. Aerobic stability was defined by the needed time (hours) for the silage temperature to rise 2 °C above the ambient during air exposure [25]. The TMR silage was sampled to determine the dynamic changes in pH, WSC, NH₃-N, organic acids, ethanol, and microbes counts by using the method mentioned above for analyses during aerobic exposure.

2.4. Statistical Analyses

The statistical analysis was conducted using IBM SPSS 27. Fermentation quality was analyzed using one-way analysis of variance (ANOVA):

$$Y_{ij} = \mu + T_i + E_{ij} \quad (2)$$

where Y_{ij} is the dependent variable; μ is overall mean; T_i is the effect of additives; and E_{ij} is the residual error term.

Aerobic stability parameters were analyzed as follows:

$$Y_{ij} = \mu + S_i + E_j + S_iE_j + \varepsilon_{ijk} \quad (3)$$

where Y_{ij} is the dependent variable; μ is overall mean; S_i is the effect of additives ($i = 1-5$); E_j is the fixed effect of time after aerobic exposure ($j = 1-4$); S_iE_j is the effect of interaction between additives and exposure days; and ε_{ijk} is the residual error term. Polynomial orthogonal contrasts were used to analyze the change in the silage parameters compared to the increasing days during aerobic exposure. The statistical difference in the data was analyzed using Tukey's multiple comparison, which was significant at the level of $p < 0.05$, with a trend being recognized at $0.05 \leq p \leq 0.10$. The Pearson correlation analyses were performed using OriginPro 2021.

3. Results

3.1. Fermentation Quality and Chemical and Microbial Compositions of Total Mixed Ration Silage

The effects of additives on the fermentation quality and the chemical and microbial composition are shown in Table 2. Compared to the control, the contents of LA, AA, and VFAs were significantly ($p < 0.001$) increased in LPB, SLNT, and SLHA silages, while the pH and AN/TN were significantly ($p < 0.001$) decreased. Among them, SLNT silage showed the highest contents of LA, AA, and VFAs, as well as the lowest pH and AN/TN. In all TMR silages, the PA and BA contents were negligible, AN/TN remained below 100 g/kg, and there was no statistical difference ($p > 0.05$) in ethanol content.

The WSC and NFC contents were significantly ($p < 0.001$) increased in all additive silages compared to CON, while the NDF contents were significantly decreased ($p < 0.05$). The SLNT silage showed the highest WSC content, with SLHA, NT, HA, and LPB following. Both the SLNT and SLHA silages showed higher ($p < 0.05$) NFC and lower ($p < 0.05$) NDF contents than other additives. All additive silages had an increased trend ($p > 0.05$) in DM and CP, and a decreased trend in ADF contents, and SLNT silage showed the highest DM and CP with the lowest ADF content. The ash and EE contents and TDN did not show a significant ($p > 0.05$) difference in all TMR silages.

For the microbial count, all additive silages significantly ($p < 0.001$) enhanced the LAB, while they decreased the AB and yeasts counts (to low or undetected levels) compared to CON. The SLNT and LPB silages showed higher ($p < 0.001$) LAB counts relative to SLHA, NT, and HA.

The Pearson correlation analysis (Figure 1) showed that WSC negatively ($p < 0.05$) correlated with pH, whereas AN, ethanol, NDF, and ADF showed positive correlations

($p < 0.05$). Significant ($p < 0.05$) positive correlations were also observed between NFC and LA, and between TDN and AA ($p < 0.05$).

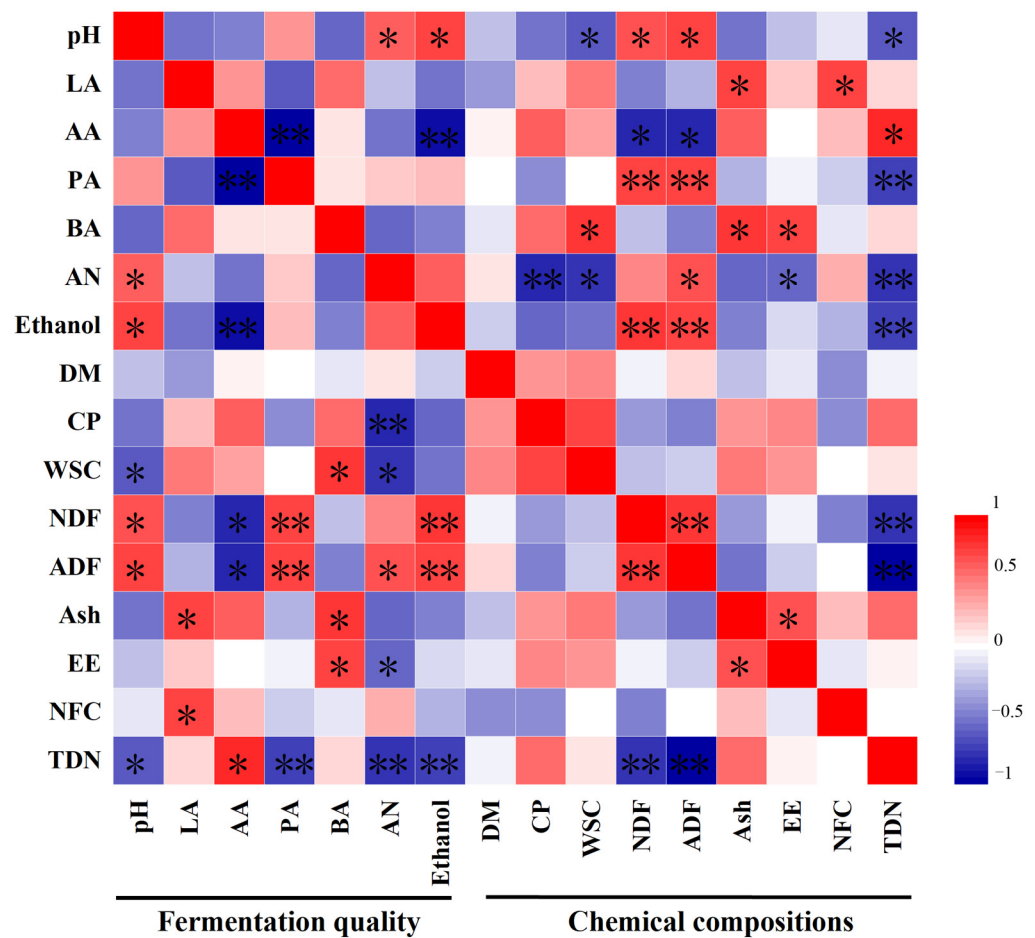


Figure 1. Pearson’s correlation heatmap of fermentation quality: chemical compositions of total mixed ration silages (* $p < 0.05$; ** $p < 0.01$). Red circles represent positive correlation, whereas blue circles represent the negative correlation. LA: lactic acid; AA: acetic acid; PA: propionic acid; BA: butyric acid; AN: ammonia nitrogen; DM: dry matter; CP: crude protein; WSC: water-soluble carbohydrate; NDF: neutral-detergent fiber; ADF: acid-detergent fiber; EE: ether extract; NFC: non-fibrous carbohydrate; TDN: total digestible nutrients.

Table 2. Fermentation quality and chemical and microbial compositions of total mixed ration silage after 45 days of ensiling.

Items ¹	Treatments ²						SEM ³	p-Value
	CON	LPB	NT	HA	SLNT	SLHA		
	<i>Fermentation characteristics (g/kg DM)</i>							
pH	5.61 ^A	4.80 ^B	5.26 ^A	5.30 ^A	4.71 ^B	4.76 ^B	0.121	<0.001
Lactic acid	27.7 ^C	37.1 ^B	29.2 ^C	28.0 ^C	44.4 ^A	41.7 ^{AB}	1.561	<0.001
Acetic acid	5.64 ^C	18.1 ^A	6.08 ^C	5.90 ^C	19.5 ^A	13.6 ^B	0.400	<0.001
Propionic acid	4.03	2.63	2.30	2.19	2.19	2.20	0.253	0.521
Propionic acid	ND	ND	ND	ND	ND	ND	0.064	0.119
Volatile fatty acids	9.67 ^C	20.73 ^A	8.38 ^C	8.09 ^C	21.7 ^A	15.8 ^B	0.329	<0.001
AN/TN (g/kg TN)	79.0 ^A	70.4 ^B	62.7 ^C	68.7 ^B	60.2 ^C	62.8 ^B	4.079	<0.001
Ethanol	12.7	8.70	8.98	9.03	8.17	8.49	2.263	0.132

Table 2. Cont.

Items ¹	Treatments ²						SEM ³	p-Value
	CON	LPB	NT	HA	SLNT	SLHA		
Chemical compositions (g/kg DM)								
Dry matter (g/kg FW)	557	561	569	566	571	568	6.222	0.653
Crude protein	230	235	245	240	255	251	5.871	0.712
Water-soluble carbohydrates	32.9 ^D	39.9 ^C	42.3 ^B	40.2 ^B	48.8 ^A	45.0 ^{AB}	1.736	<0.001
Neutral-detergent fiber	349 ^A	321 ^B	321 ^B	325 ^B	294 ^C	301 ^C	7.523	<0.001
Acid-detergent fiber	210	211	215	216	197	203	7.149	0.811
Ash	87.3	86.2	86.1	85.7	86.0	86.6	1.363	0.641
Ether extract	40.4	41.9	42.4	41.1	44.7	43.6	1.202	0.081
Non-fibrous carbohydrate	291 ^C	316 ^{AB}	304 ^B	308 ^B	321 ^A	321 ^A	8.633	0.005
TDN (%DM)	68.0	67.6	67.9	67.7	68.9	68.5	1.066	0.079
Microbial compositions (log₁₀ cfu/g FW)								
Lactic acid bacteria	6.90 ^C	8.44 ^A	7.56 ^B	7.19 ^B	8.49 ^A	7.90 ^B	0.349	<0.001
Aerobic bacteria	5.87 ^A	3.47 ^B	<2.00	<2.00	<2.00	<2.00	1.132	<0.001
Yeasts	3.13 ^A	<2.00	<2.00	<2.00	<2.00	<2.00	0.833	<0.001

Values in the same row with different superscript letters (A–D) are significantly different ($p < 0.05$). ¹ DM: dry matter; AN/TN: ammonia nitrogen/total nitrogen; FW: fresh weight; TDN: total digestible nutrients; Log₁₀: decimal logarithm; cfu: colony-forming units. ² CON: control; LPB: lactic acid bacteria (*Lactiplantibacillus plantarum* + *Lentilactobacillus buchmeri*); NT: natamycin; HA: hexanoic acid; SLNT: lactic acid bacteria + natamycin; SLHA: lactic acid bacteria + hexanoic acid. ³ SEM: standard error of the mean.

3.2. Aerobic Stability of Total Mixed Ration Silage

Table 3 illustrates the changes in fermentation quality during aerobic exposure. During aerobic exposure, additives, aerobic exposure days, and their interaction significantly ($p < 0.001$) affected pH, LA, and AA contents, whereas they had no significant impact ($p > 0.05$) on PA and ethanol contents. In all treatment silages, the contents of LA decreased linearly ($p < 0.001$), while the pH increased linearly ($p < 0.001$). The AA content exhibited a quadratic decrease ($p < 0.05$) as exposure days increased. Compared to the control, all silages treated with additives resulted in a significant ($p < 0.001$) increase in LA content and a decrease ($p < 0.001$) in pH, particularly SLNT and SLHA. The LPB, SLNT, and SLHA significantly ($p < 0.001$) increased the AA contents as compared with other additives, and the SLNT silage had the highest AA content.

Table 3. Changes in fermentation quality of total mixed ration silage after exposure to air.

Items	Treatments ¹	Aerobic Exposure Days (d)				SEM ²	p-Value ³			Model Construction p ⁴	
		0	2	4	6		T	D	T × D	L	Q
pH	CON	5.61 ^{Ac}	6.27 ^{Ab}	7.35 ^{Aa}	7.50 ^{Aa}	0.091	<0.001	<0.001	<0.001	<0.001	0.005
	LPB	4.80 ^{Bc}	4.86 ^{Dc}	5.21 ^{Db}	6.77 ^{Ca}	0.046				<0.001	<0.001
	NT	5.26 ^{Ac}	5.39 ^{Cc}	6.48 ^{Cb}	7.23 ^{Ba}	0.067				<0.001	<0.001
	HA	5.30 ^{Ac}	5.55 ^{Bc}	6.88 ^{Bb}	7.48 ^{Aa}	0.109				<0.001	0.008
	SLNT	4.71 ^{Bc}	4.73 ^{Dc}	4.98 ^{Db}	6.37 ^{Da}	0.080				<0.001	<0.001
	SLHA	4.76 ^{Bb}	4.81 ^{Db}	5.05 ^{Db}	6.48 ^{Da}	0.037				<0.001	<0.001
	SEM ²	0.121	0.133	0.059	0.063						
	p-value	<0.001	<0.001	<0.001	<0.001						
Lactic acid (g/kg DM)	CON	27.7 ^{Ca}	18.1 ^{Db}	10.7 ^{Dc}	5.41 ^{Dd}	0.695	<0.001	<0.001	<0.001	<0.001	0.002
	LPB	37.1 ^{Ba}	33.6 ^{Ba}	25.8 ^{Bb}	13.1 ^{Bc}	0.999				<0.001	<0.001
	NT	29.2 ^{Ca}	25.2 ^{Ca}	19.4 ^{Cb}	8.86 ^{Cc}	1.059				<0.001	<0.001
	HA	28.0 ^{Ca}	21.4 ^{CDa}	12.3 ^{Dc}	8.36 ^{CDc}	1.267				<0.001	0.066
	SLNT	44.4 ^{Aa}	39.9 ^{Aab}	32.9 ^{Ab}	24.9 ^{Ac}	1.895				<0.001	0.011
	SLHA	41.7 ^{ABa}	38.6 ^{Aa}	31.2 ^{ABb}	21.0 ^{Ac}	2.043				<0.001	0.175
	SEM ²	1.561	1.406	1.636	0.940						
	p-value	<0.001	<0.001	<0.001	<0.001						

Table 3. Cont.

Items	Treatments ¹	Aerobic Exposure Days (d)				SEM ²	p-Value ³			Model Construction p ⁴	
		0	2	4	6		T	D	T × D	L	Q
Acetic acid (g/kg DM)	CON	5.64 ^C	4.19 ^C	5.35 ^B	4.44 ^B	0.278	<0.001	<0.001	<0.001	0.313	0.201
	LPB	18.1 ^{Aa}	14.6 ^{Ab}	10.3 ^{Abc}	11.3 ^{Ac}	0.420				0.708	<0.001
	NT	6.08 ^C	5.34 ^C	6.24 ^B	6.58 ^B	0.219				0.001	0.305
	HA	5.90 ^C	5.06 ^{Ca}	5.46 ^B	5.44 ^B	0.196				0.049	0.061
	SLNT	19.5 ^{Aa}	15.4 ^{Ab}	11.6 ^{Ac}	12.7 ^{Abc}	0.291				<0.001	0.001
	SLHA	13.6 ^{Ba}	10.4 ^{Ba}	8.23 ^{Ab}	10.5 ^{Aa}	0.249				<0.001	<0.001
	SEM ²	0.400	0.348	0.131	0.150						
p-value	<0.001	<0.001	0.090	0.204							
Propionic acid (g/kg DM)	CON	4.03	5.85	6.82	5.88	0.094	0.272	0.241	0.379	0.389	0.052
	LPB	2.63	3.89	4.83	4.77	0.310				0.567	0.653
	NT	2.30	3.97	4.77	5.03	0.136				0.763	0.305
	HA	2.19	3.97	4.86	4.89	0.079				0.614	0.609
	SLNT	2.19	3.74	4.66	4.93	0.089				0.189	0.077
	SLHA	2.20	3.73	4.62	4.98					0.177	0.213
	SEM ²	0.253	0.123	0.061	0.127						
p-value	0.521	0.263	0.109	0.446							
Ethanol (g/kg DM)	CON	12.7	11.4	8.34	5.97	1.051	0.371	0.315	0.235	0.301	0.086
	LPB	8.70	6.93	5.23	3.19	1.458				0.547	0.819
	NT	8.98	7.04	4.10	2.60	0.609				0.261	0.632
	HA	9.03	7.61	4.98	3.20	0.612				0.182	0.451
	SLNT	8.17	6.60	3.38	3.00	0.519				0.781	0.503
	SLHA	9.49	7.65	4.95	3.31	0.541				0.605	0.737
	SEM ²	2.264	0.600	0.241	0.330						
p-value	0.134	0.238	0.301	0.712							

Values in the same row with different superscript letters (A–D) are significantly different ($p < 0.05$). Values in the same column with different lowercases (a–d) are significantly different ($p < 0.05$). ¹ CON: control; LPB: lactic acid bacteria (*Lactiplantibacillus plantarum* + *Lentilactobacillus buchneri*); NT: natamycin; HA: hexanoic acid; SLNT: lactic acid bacteria + natamycin; SLHA: lactic acid bacteria + hexanoic acid. ² SEM: standard error of the mean. ³ T: effect of treatment; D: effect of exposure day; T × D: effect of treatment and exposure day interactions. ⁴ L and Q: linear and quadratic effect of exposure days.

Table 4 illustrates the chemical and microbial compositions during aerobic exposure. Additives, aerobic exposure days, and their interaction significantly ($p < 0.05$) influenced WSC content, AN/TN, LAB, and aerobic bacteria and yeasts counts. During aerobic exposure, the AN/TN and counts of AB and yeasts showed a significant linear increase ($p < 0.05$), while the WSC content and LAB count showed a significant linear decrease ($p < 0.05$). On the sixth day of aerobic exposure, all additives showed an AN/TN content below 100 g/kg TN. The SLNT exhibited the greatest ($p < 0.001$) WSC content and the lowest ($p < 0.001$) AN/TN among all additive silages. In comparison to the control, all additives showed significantly ($p < 0.001$) higher counts of LAB and lower counts of aerobic bacteria and yeasts ($p < 0.001$); the SLNT silage consistently maintained the highest LAB and the lowest aerobic bacteria and yeast counts during aerobic exposure.

Figure 2 illustrates the temperature changes and aerobic stability during aerobic exposure. Compared to the control, all additives were relatively stable (Figure 2A). The aerobic stability of the TMR silage was significantly increased by all additive silages ($p < 0.001$) (Figure 2B). SLNT showed the best ($p < 0.001$) aerobic stability for 85.5 h, followed by SLHA, NA, HT, and LPB, which were maintained for 77.5, 65, 54.5, and 43 h, respectively.

Table 4. Changes in chemical and microbial compositions of total mixed ration silage after exposure to air.

Items ¹	Treatments ²	Aerobic Exposure Days (d)				SEM ³	p-Value ⁴			Model Construction p ⁵	
		0	2	4	6		T	D	T × D	L	Q
WSC (g/kg DM)	CON	32.9 ^{Ca}	27.0 ^{Da}	20.4 ^{Cb}	13.4 ^{Bd}	1.301	<0.001	<0.001	<0.001	<0.001	0.653
	LPB	39.9 ^{Ca}	30.6 ^{CDb}	25.0 ^{BCc}	17.6 ^{Bc}	1.614				<0.001	0.036
	NT	42.3 ^{ABa}	35.4 ^{BCa}	27.1 ^{BCb}	20.6 ^{Bc}	1.571				<0.001	0.133
	HA	40.3 ^{BCa}	32.6 ^{CDb}	26.6 ^{BCc}	19.4 ^{Bc}	1.402				<0.001	0.073
	SLNT	48.8 ^{Aa}	43.0 ^{Aab}	39.6 ^{Ab}	32.8 ^{Ac}	1.425				<0.001	0.046
	SLHA	45.0 ^{ABa}	37.8 ^{Bb}	31.6 ^{Bb}	25.3 ^{Bc}	2.189				<0.001	0.042
	SEM ³	1.733	1.308	1.737	1.628						
	p-value	<0.001	<0.001	<0.001	<0.001						
AN/TN (g/kg TN)	CON	79.0 ^{Ac}	82.6 ^{Abc}	87.9 ^{Ab}	108 ^{Aa}	5.073	0.005	<0.001	<0.001	<0.001	0.395
	LPB	70.4 ^{ABa}	74.4 ^{Bab}	80.6 ^{Bb}	89.2 ^{Ba}	6.016				<0.001	0.013
	NT	62.7 ^{Bb}	66.8 ^{Cab}	75.4 ^{Ca}	86.4 ^{Ba}	4.664				<0.001	0.661
	HA	68.7 ^{Bc}	73.1 ^{Ac}	78.7 ^{Ab}	87.5 ^{Ba}	5.535				<0.001	0.938
	SLNT	60.2 ^{Bb}	62.3 ^{Cab}	66.4 ^{Dab}	72.8 ^{Da}	5.490				0.001	0.014
	SLHA	62.8 ^{Cc}	63.4 ^{Cc}	70.0 ^{Cb}	81.1 ^{Ca}	5.400				0.012	0.034
	SEM ³	4.079	3.116	3.116	5.557						
	p-value	<0.001	<0.001	<0.001	<0.001						
LAB (log ₁₀ cfu/g FW)	CON	6.90 ^{Ca}	6.51 ^{Cb}	5.75 ^{Cc}	4.35 ^{Cd}	0.933	<0.001	<0.001	<0.001	<0.001	0.001
	LPB	8.44 ^{Aa}	8.18 ^{Aa}	7.75 ^{Ab}	6.86 ^{Ac}	0.230				<0.001	0.001
	NT	7.56 ^{Ba}	7.51 ^{Bb}	6.75 ^{Bc}	5.65 ^{Bd}	0.170				0.002	0.261
	HA	7.19 ^{Ba}	6.82 ^{Cb}	6.29 ^{Bc}	5.18 ^{Bd}	0.764				<0.001	0.061
	SLNT	8.56 ^{Aa}	8.43 ^{Aa}	7.84 ^{Ab}	7.09 ^{Ac}	0.194				0.004	0.056
	SLHA	7.90 ^{Ba}	7.71 ^{Aa}	7.01 ^{Ab}	6.27 ^{Ac}	0.303				0.031	0.753
	SEM ³	1.349	1.530	1.347	1.333						
	p-value	<0.001	<0.001	<0.001	<0.001						
AB (log ₁₀ cfu/g FW)	CON	5.87 ^{Ad}	6.36 ^{Ac}	7.13 ^{Ab}	8.06 ^{Aa}	0.442	<0.001	<0.001	<0.001	<0.001	0.036
	LPB	3.47 ^{Cc}	5.01 ^{Bb}	5.38 ^{Bb}	6.08 ^{Ba}	0.510				0.004	1.198
	NT	<2.00	4.60 ^{Bb}	5.05 ^{Cb}	5.53 ^{Ca}	0.612				<0.001	0.031
	HA	<2.00	4.81 ^{Cc}	5.31 ^{Ba}	5.66 ^{Ba}	0.510				<0.001	0.163
	SLNT	<2.00	4.47 ^{Bc}	4.89 ^{Cb}	5.29 ^{Ca}	0.476				0.001	0.754
	SLHA	<2.00	4.53 ^{Dc}	4.99 ^{Cb}	5.42 ^{Ba}	0.748				<0.001	0.147
	SEM ³	1.132	0.526	0.485	0.404						
	p-value	<0.001	<0.001	<0.001	<0.001						
Yeasts (log ₁₀ cfu/g FW)	CON	3.13 ^{Ad}	3.45 ^{Ac}	3.96 ^{Ab}	4.75 ^{Aa}	1.012	0.008	<0.001	<0.001	<0.001	0.044
	LPB	<2.00	2.3 ^{Bb}	3.07 ^{Bb}	3.52 ^{Ba}	0.408				<0.001	0.791
	NT	<2.00	<2.00	2.47 ^{Cb}	3.13 ^{Ca}	0.348				<0.001	0.358
	HA	<2.00	<2.00	2.83 ^{Bb}	3.69 ^{Ba}	0.650				<0.001	0.736
	SLNT	<2.00	<2.00	<2.00	2.60 ^{Ca}	0.363				0.001	0.075
	SLHA	<2.00	<2.00	2.53 ^{Bb}	3.69 ^{Ba}	0.801				0.002	0.244
	SEM ³	0.833	0.572	0.653	0.539						
	p-value	<0.001	<0.001	<0.001	<0.001						

Values in the same row with different superscript letters (A–D) are significantly different ($p < 0.05$). Values in the same column with different lowercases (a–d) are significantly different ($p < 0.05$). ¹ WSC: water-soluble carbohydrate; AN/TN: ammonia nitrogen/ total nitrogen; LAB: lactic acid bacteria; AB: aerobic bacteria; Log₁₀: decimal logarithm; cfu: colony-forming unit. ² CON: control; LPB: lactic acid bacteria (*Lactiplantibacillus plantarum* + *Lentilactobacillus buchneri*); NT: natamycin; HA: hexanoic acid; SLNT: lactic acid bacteria + natamycin; SLHA: lactic acid bacteria + hexanoic acid. ³ SEM: standard error of the mean. ⁴ T: effect of treatment; D: effect of exposure day; T × D: effect of treatment and exposure day interactions. ⁵ L and Q: linear and quadratic effect of exposure days.

3.3. Relationship between Acetic Acid with Water-Soluble Carbohydrate and Ethanol during Aerobic Exposure

As illustrated in Figure 3, the relationship between acetic acid content (x) and WSC content (y) during aerobic exposure was optimally modeled using a positive polynomial method ($y = -30.86 + 11.63x - 0.48x^2$, $R^2 = 0.72$, $RMSE = 12.19$, $p < 0.05$, $n = 24$). Acetic acid (x) with ethanol was best modeled using an inverse linear method ($y = -1.42x + 21.44$, $R^2 = 0.84$, $RMSE = 3.36$, $p < 0.05$, $n = 24$).

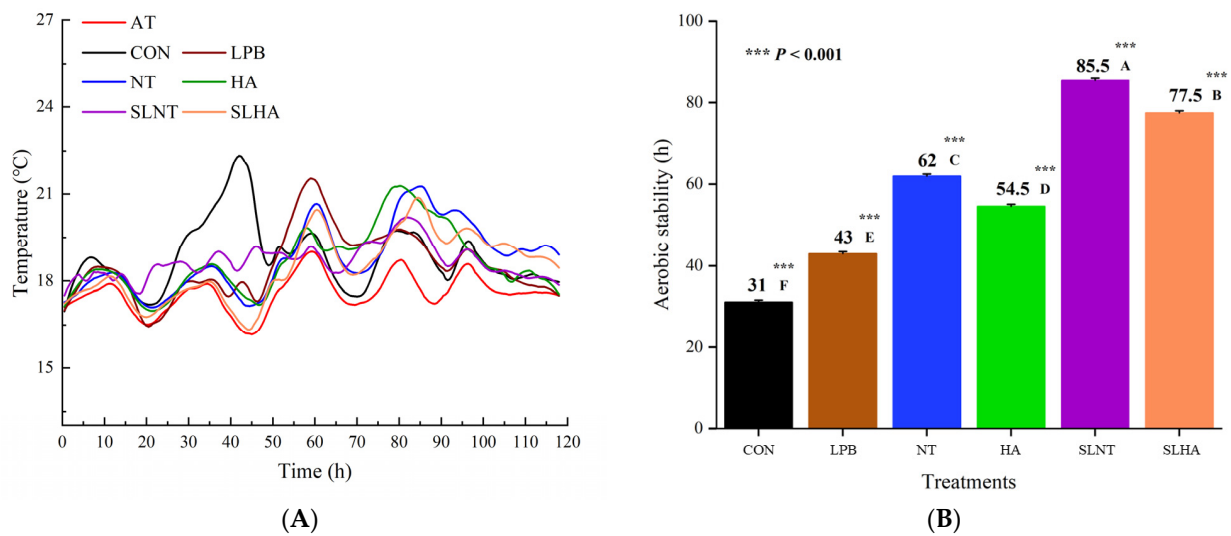


Figure 2. Effect of additives on temperature changes (A) and aerobic stability (B) of total mixed ration silages during air exposure. Different letters (A–F) in (B) are considered as significant among treatments (***p* < 0.001), and vertical bars represent the standard deviations of the averages. CON: control; LPB: lactic acid bacteria (*Lactiplantibacillus plantarum* + *Lentilactobacillus buchneri*); NT: natamycin; HA: hexanoic acid; SLNT: lactic acid bacteria + natamycin; SLHA: lactic acid bacteria + hexanoic acid.

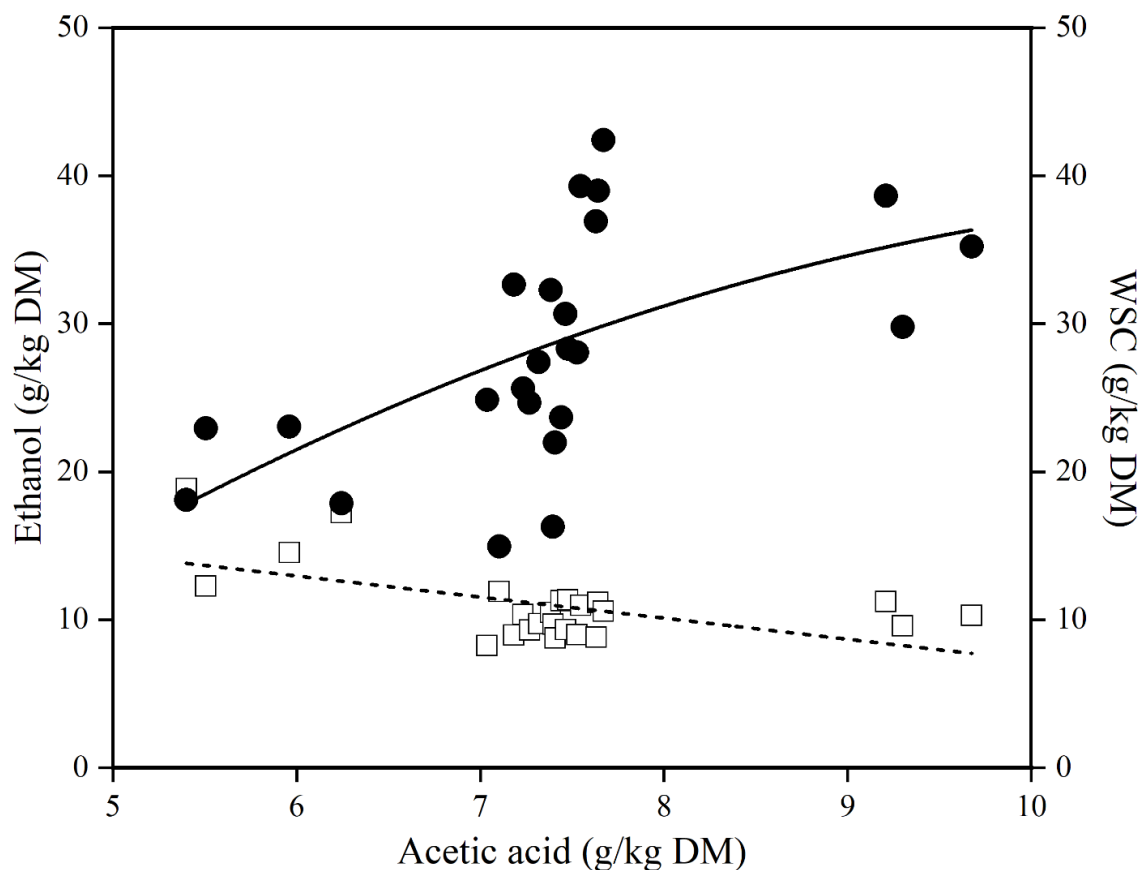


Figure 3. Relationship between the acetic acid content and WSC content (solid line, $y = -30.86 + 11.63x - 0.48x^2$, $R^2 = 0.72$, $RMSE = 12.19$, $p < 0.05$, $n = 24$) and the ethanol content (dashed line, $y = -1.42x + 21.44$, $R^2 = 0.84$, $RMSE = 3.36$, $p < 0.05$, $n = 24$), respectively, in total mixed ration silage treated with different additives during air exposure. WSC: water-soluble carbohydrate.

4. Discussion

It is well-known that quality silage depends on a sufficient WSC (>60 g/kg DM) content and LAB (>10⁵ cfu/g FW) count, and a low BC. This work showed a favorable WSC (83.87 g/kg DM) and LAB count (>10⁶ cfu/g FW) in TMR. However, the high AB and yeast counts (>10⁵ cfu/g FW) pose high risks to fermentation quality and aerobic stability [26]. Consequently, quality TMR silage-making without additives is a formidable challenge.

Our previous research demonstrated that *Lactiplantibacillus plantarum* improved lactic acid fermentation efficiency, and that natamycin and hexanoic acid had antifungal benefits, each of them improving fermentation quality. Furthermore, their combination showed a superior synergistic effect for improving fermentation quality and aerobic stability. As anticipated, there were higher LA and acetic acid AA contents in LPB-treated silage (LPB, SLNT, and SLHA) than other additives, reflecting the characteristic activities of LAB. *L. plantarum*, a homofermentative lactic acid bacterium (LAB), rapidly produced LA via the homofermentative Embden–Meyerhof pathway during ensiling. In contrast, *L. buchneri*, a heterofermentative LAB, predominantly produced acetic acid (AA) through heterofermentative glucose metabolism. When the substrates reached sufficiency, *L. plantarum* efficiently produced a large quantity of LA along with a quick pH drop, causing homolactic fermentation in silages; the presence of *L. buchneri* led to significant acetic acid production through heterolactic fermentation [11]. The association of LAB with natamycin (SLNT) accelerated the LA and AA accumulation compared to the other treatments during ensiling. This might be due to natamycin's antifungal properties effectively suppressing yeast activity, preserving more fermentable substrates for LAB. Moreover, this demonstrated that natamycin would not impair LAB activity, and their combination could be the powerful strategy for quality silage-making. Similarly, Shah et al. [27] also found that natamycin-treated silages showed a greater LA and AA accumulation than the control (without natamycin).

The propionic, butyric acid, and ammonia nitrogen contents were detrimental factors during ensiling [28]. In this work, propionate and butyrate were negligible in all TMR silages, and the AN/TN content was much lower than 100 g/kg TN, indicating that all the TMR silages fermented well and were effectively preserved [29]. Furthermore, there was lower AN/TN in the additives than in the control, which may be attributed to the bacteriostatic and bactericidal action of LAB, natamycin, and hexanoic acid. Natamycin, a polyene macrolide antibiotic, effectively targets yeasts and molds [30]. Hexanoic acid destabilizes bacterial cell membranes and disrupts oxidative phosphorylation by interfering with the electron transport chain, leading to membrane disruption [15]. Koç et al. [31] reported that the low pH condition during ensiling would lead to a reduction in ammonia nitrogen concentrations.

All additive silages showed higher residual WSC and CP contents, which can be attributed to beneficial acidic environments and antibacterial activity in antagonizing undesirable bacteria during ensiling [11]. Particularly, SLNT silages had the highest WSC and CP contents and the lowest aerobic bacteria and yeasts counts compared to other additive silages, which was the result of the robust combined antimicrobial effects of LAB and natamycin [32].

Non-fiber carbohydrates (NFC) are crucial in ruminant nutrition by offering adequate energy for efficient microbial protein synthesis [33]. Providing silage with sufficient NFC contents for ruminants not only enhances synthesis efficiency and minimizes nitrogen losses but also contributes to increased feed intake [34]. The higher NFC contents in additive silages were primarily attributed to their decreased NDF content, and the lower NDF content may be associated with their relatively low pH promoting the hydrolysis of structural carbohydrates. These results implied SLNT silage had the highest feeding value, followed by SLHA, NT, HA, and CON silages.

The Pearson correlation analysis demonstrated a negative correlation between WSC and pH, mainly due to the inhibition of WSC consumption; the fermentation products (primarily LA) break down structural carbohydrates into WSC via acid hydrolysis. These findings were consistent with those reported in a prior study conducted by Kung, Shaver,

Grant, and Schmidt [8]. Conversely, a positive correlation between AN, ethanol, NDF, and ADF with pH was observed, which might be due to a low pH suppressing undesirable microorganisms and increasing the acid degradation of structural carbohydrates. Xu et al. [35] also observed that the pH had the same negative correlation with the AN and ethanol of TMR silages. Furthermore, LA and AA were positively correlated to the NFC and TDN, indicating that their antimicrobial effects contribute to less nutrient loss.

Minimizing silage spoilage during aerobic exposure is a major challenge for dairy farmers [27]. When air infiltrates silage, yeasts (mainly lactate-assimilating yeasts) start to proliferate; silage is prone to deterioration, which leads to a temperature and pH increase, and nutrient losses. Moreover, the growth of other undesirable bacteria further exacerbate spoilage, ultimately resulting in a decrease in animal production efficiency.

During aerobic exposure, the pH linearly increased while the LA content linearly decreased, which attributed to the consumption of LA by lactate-assimilating yeasts [9]. The AA content slightly increased during the later stages of aerobic exposure, which might be due to acetobacter bacteria oxidizing lactate and ethanol into acetate, CO₂, and water in an aerobic environment [36,37]. Additives, particularly SLNT and SLHA, delayed the LA decrease and pH increase more than CON. This was because the production of organic acids, especially acetic acid, work synergistically with natamycin and hexanoic acid, effectively restricting the proliferation of aerobic microorganisms that contribute to the deterioration process [38]. Furthermore, the higher AA content in SLNT silage relative to SLHA silage might be attributed to the suppression of *L. buchneri* counts by hexanoic acid. Wang et al. [39] also found that hexanoic acid decreased AA content, and they attributed this to the suppression of *L. buchneri* by hexanoic acid.

The stability of all the additive silages was greater than that of the control, as indicated by the higher levels of WSC and the lower AN/TN contents and counts of AB and yeast. SLNT silage was the most stable (85.5 h), followed by SLHA, NT, HA, and LPB, indicating the greater synergism of LAB with natamycin relative to LAB with hexanoic acid. This was because the antimicrobial actions of hexanoic acid (pKa 4.88) were diminished as the pH sharply increased (>5.2) during the last four days of aerobic exposure. Consequently, natamycin (effective at pH 5.0–9.0) was more stable, showing potent antimicrobial activity compared to hexanoic acid under a high pH environment during aerobic exposure [6,40]. Natamycin disrupts yeast growth and triggers cell death via ergosterol interaction without cell membrane permeabilization [41]. In contrast, hexanoic acid interferes with the mitochondrial membrane potential, leading to cell death [15]. The LAB, working synergistically with natamycin, had a significant synergistic effect on the antimicrobial effects [13,30].

The characteristics of acetic acid, a beneficial short-chain fatty acid with antifungal properties in aerobic deterioration [42], were confirmed by a strong negative linear correlation with ethanol content in our study ($R^2 = 0.84$, $p < 0.05$). The decrease in ethanol content was primarily because acetic acid from LAB effectively inhibits yeast activity during aerobic exposure. Schmidt and Kung [43] also detected the high inverse correlation between the AA content and yeasts after the exposure of the silages to air. Meanwhile, a positive relation between the AA and the WSC was observed ($R^2 = 0.72$, $p < 0.05$), suggesting that acetic acid effectively inhibits the metabolism of WSC via undesirable microorganisms during aerobic exposure.

5. Conclusions

Compared to the control, the additives significantly increased the LA and AA contents, the LAB counts, and decreased the pH and NH₃-N. In terms of aerobic stability, the additive silages were more stable, characterized by significantly prolonged hours of aerobic stability, a delayed pH increase, an LA and WSC decrease, and minimized yeast proliferation. Among the treatments, lactic acid bacteria + natamycin (SLNT) silage showed the best stability, followed by lactic acid bacteria + hexanoic acid (SLHA), natamycin (NA), hexanoic acid (HA), and lactic acid bacteria (LPB). These findings suggest that SLNT should be recommended as the optimal additive in improving the fermentation quality and aerobic

stability of TMR silage. However, the effects of additives on growth performance, nutrient digestibility, and carcass traits need further investigation.

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